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Award Number: DAMD17-01-1-0308

TITLE: Mammaglobin and Lipophilin Related Molecules in Normal and Tumor Human Breast Tissue: Expression, Hormone Regulation and Functional Analysis

PRINCIPAL INVESTIGATOR: Etienne R. Leygue, Ph.D.

CONTRACTING ORGANIZATION: University of Manitoba
Winnipeg, Manitoba, Canada R3E 0W3

REPORT DATE: August 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2004	Annual Summary (1 Aug 01 - 31 Jul 04)

4. TITLE AND SUBTITLE
Mammaglobin and Lipophilin Related Molecules in Normal and Tumor Human Breast Tissue: Expression, Hormone Regulation and Functional Analysis

5. FUNDING NUMBERS
DAMD17-01-1-0308

6. AUTHOR(S)
Etienne R. Leygue, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Manitoba
Winnipeg, Manitoba, Canada R3E 0W3

E-Mail: eleygue@cc.umanitoba.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Genes, expression of which is breast specific or is altered during breast tumorigenesis, represent potential targets for new preventive and curative strategies. Such genes, Mammaglobin (**MGB1**), **hSBEM** (Human Small Breast Epithelial Mucin), **Psoriasin**, Estrogen receptor beta (**hERB**) and **SRA** (Steroid receptor RNA activator), have been studied during the time of this Award (July 2001-July 2004). We found that:

MGB1 expression correlates with ER (an established breast cancer prognostic and predictive factor), but is not modified by hormone treatments, suggesting it might be a new independent breast cancer prognostic marker.

hSBEM, we identified a new breast specific gene which represents an attractive candidate for a new breast tumor marker with obvious potential for cancer diagnostics and treatment

SRA, previously believed to belong to the family of non-coding RNA and able to activate steroid receptor activity, is indeed translated into a protein which we suspect also interfers with estrogen signaling pathway.

Psoriasin, highly expressed in high risk DCIS, can contribute to breast tumor progression through modulation of Jab1 activity and is a target gene of ER-beta.

hERB isoforms have promoter-specific differential activities and modulate the response of breast cancer cells to antiestrogen treatment. this suggests that these isoforms may have a role in differentially modulating estrogen action during breast tumor progression.

14. SUBJECT TERMS
Mammaglobin, Sra, Estrogen Receptor Beta, Co-Activator, SBEM, Psoriasin

15. NUMBER OF PAGES
146

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Breast cancer remains one of the most frequently diagnosed cancers today. One in eight women is expected to present with breast cancer within her lifetime in developed countries. An estimated 1,000,000 cases are detected each year worldwide and in Canada alone, an estimated 21,200 women will be diagnosed with breast cancer and 5,300 will be lost to this disease in 2003 (1). For women with recurrent disease, the median time of survival is about two years (2). Despite such striking statistics, breast cancer related mortality is slowly decreasing as continuing research has led to earlier detection, more treatment options for breast cancer patients and an improved chance of long-term survival. Improving the diagnosis and clinical management of breast cancer requires access to and characterization of biomarkers that are able to reflect the molecular phenotype of breast tissue. Genes, expression of which is breast specific or is altered during breast tumorigenesis, represent potential targets for new preventive and curative strategies. Such genes, Mammaglobin (**MGB1**), **hSBEM** (Human Small Breast Epithelial Mucin), **Psoriasin**, Estrogen receptor beta (**hERB**) and **SRA** (Steroid receptor RNA activator) have been studied in our group during the period covered by this training award (July 2001-July 2004). The results obtained are summarized below.

BODY

MGB1: Mammaglobin A was first identified in 1996, as a breast specific member of the uteroglobin gene family overexpressed in some breast tumors (3, 4). As shown in **APPENDIX 1**, we evaluated MGB1 expression at the mRNA and at the protein level by reverse-transcription polymerase chain reaction and immunocytochemistry in 52 and 32 breast tumors, respectively (5). Both MGB1 mRNA and protein expression were significantly higher in estrogen receptor positive compared to estrogen negative tumors (Mann-Whitney rank sum test, $p = 0.04$; Chi-square test, $p = 0.01$; respectively). In contrast, MGB1 expression did not correlate with progesterone receptor levels or Notthingham grade. As estrogen and antiestrogen treatment of estrogen positive breast cancer cell lines does not modify MGB1 expression we suggest that MGB1 could be a new independent breast cancer prognostic marker.

hSBEM: We have identified a novel putative breast-specific gene (hSBEM, Human Small Breast Epithelial Mucin), which represents an attractive candidate for a new breast tumor marker with obvious potential for cancer diagnostics (6, **APPENDIX 2, APPENDIX 3**). We have now obtained mammary cells MCF-7 stably expressing V5-tagged hSBEM protein as well as antibodies recognizing the transfected as well as the endogenous protein. As underlined in **APPENDIX 4**, we have now characterized the expression of the hSBEM protein in breast cancer cell lines and in a series of breast tumors. Our preliminary data suggest that a inverse correlation might exist between SBEM protein expression and estrogen receptor levels. As reported in **APPENDIX 5**, we are currently investigating the promoter region of the hSBEM gene in order to identify elements responsible for its breast specific expression. Our preliminary data identified a region of 87bp, located

between nucleotides -357/-270 upstream of the initiating methionine codon, which is sufficient to induce a strong expression of the luciferase reporter gene in three different mammary cell lines but in none of the three non breast cancer cell lines studied.

Psoriasin: We have previously identified psoriasin (S100A7) as a differentially expressed gene between DCIS and invasive breast carcinoma (7). In collaboration with Dr. Watson, we have found that psoriasin physically interacts with Jab1 (c-jun activation-domain binding protein 1) and we suggest that intracellular psoriasin influences breast cancer progression through stimulation of Jab1 activity (8, **APPENDIX 6**).

hERB: Estrogens regulate the growth and development of normal human mammary tissue and are also involved in breast tumor progression. Estrogen action is mediated mainly through two estrogen receptors (ERs): ER-alpha (9) and ER-β1 (10, 11). Several variant forms of ER-β1 have been identified in breast tissues (for reviews see Refs. 12-15). In collaboration with Dr. Murphy, we investigated the putative functional characteristics of human receptor beta isoforms (16, **APPENDIX 7**). We showed that only ER- β1 was able to bind ligand whereas all ER-beta isoforms bind to DNA even though their binding abilities differ. ER-beta isoforms inhibition of ER-alpha and ER-β1 transcriptional activity is promoter specific. Overall, our data suggest that ER-beta isoforms may have a differentially modulating estrogen action. As shown in **APPENDIX 8** we also found that the expression of hERβ isoforms differ in primary tumors of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy suggesting a possible role of ERβ related protein in hormone resistance (17). In order to better understand the role of ER-β1 on estrogen and antiestrogen action, we have also generated a stable MCF-7 breast cancer cell line expressing ER-β1 under the control of an inducible Tet-on system (**APPENDIX 9**). We found that the overexpression of ER-β1 in these cells was leading to a higher sensitivity to Tamoxifen. Interestingly, we also found that Psoriasin, was a possible downstream marker of ER-β1 activity in breast cancer cells (**APPENDIX 10**).

SRA: SRA is a steroid receptor co-activator acting as a functional RNA and still classified as belonging to the growing family of functional non-coding RNAs (18). None of the different SRA transcripts originally described encoded a detectable SRA protein following in vitro and in vivo translation experiments. We have previously shown that SRA RNA was differentially expressed in normal and tumor breast tissues (19, 20). We have now identified three new SRA-RNA isoforms differing mainly from the originally cloned SRA by an extended 5 ' extremity. These long SRA isoforms, able to encode a stable protein in vitro, led to the production in vivo of a nuclear protein when transfected into the MCF-7 human breast cancer cell line. Reverse-transcription polymerase chain reaction and Western blot analysis of RNA and protein extracts from different breast cancer cell lines confirmed the presence of endogenous coding SRA isoforms and their corresponding proteins. Our results demonstrate that full-length SRA-RNAs likely to encode stable proteins are widely expressed in breast cancer cell lines (21, **APPENDIX 11**). Using database search, RNAi technology and Western blot, we have now definitively established the existence of the SRA protein (SRAP) and its

conservation in chordata (**APPENDIX 12, 13**). We have now obtained several clones of MCF-7 breast cancer cells stably expressing the SRAP protein. Our preliminary data suggest that estrogen signaling pathway is modified in these cells (**APPENDIX 14**). Moreover, we have now correlated the expression of this SRAP protein in primary breast tumors with a better chance for the patients to survive under tamoxifen treatment. Overall, these data suggest a possible role of the SRAP in estrogen signaling pathway.

KEY RESEARCH ACCOMPLISHMENTS

- We hypothesized that MGB1 could be a newindependent prognostic marker of breast cancer.
- We obtained breast cancer cells stably expressing tagged hSBEM protein.
- We obtained antibodies recognizing both transfected and endogenous hSBEM protein.
- We found 87bp in the hSBEM promoter which could be responsible for its breast specific expression.
- We established a possible mechanism of action for psoriasin in breast tissues.
- We confirmed that hERB isoforms are likely to play a role in response to hormone therapy.
- We have identified psoriasin as a potential target gene of ERbeta1
- We have identified new SRA isoforms encoding stable SRAP proteins.
- We established definitively the existence of the SRAP protein.
- We have therefore identified the first functional RNA coding a protein.
- We obtained breast cancer cells stably expressing tagged SRAP proteins.
- We obtained antibodies recognizing transfected and endogenous SRA proteins
- We found that the stable transfection of SRAP was decreasing the ability for cells to answer to estrogens.
- We found a relationship between SRAP detection in breast tumors and patients survival.

REPORTABLE OUTCOMES

* Seven articles published (APPENDICES 1, 2, 6, 7, 8, 11, 12) and two submitted (APPENDICES 3, 9)

CONCLUSION

Two projects are currently funded within the laboratory: SRA and hSBEM. We are now investigating the function and role of SRAP in breast tumorigenesis and the possible use of hSBEM to target/detect breast cancer cell in vivo.

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Appendices

Appendix 1 Endocrine. 2003. 21: 245-250

Endocrine, vol. 21, no. 3, 245-250 August 2003 0969-711X/03/21:245-250/$25.00

Relationship Between Mammaglobin Expression and Estrogen Receptor Status In Breast Tumors

Xiao-feng Guan,[1] Mohammad K. Hamedani,[1] Adewale Adeyinka,[2] Christina Walker,[1] Angela Kemp,[1] Leigh C. Murphy,[1] Peter H. Watson,[2] and Etienne Leygue[1]

[1]*Departments of Biochemistry and Medical Genetics; and* [2]*Pathology, University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, Canada, R3EOW3*

Mammaglobin (SCGB2A2) is a breast-specific member of the secretoglobin (SCGB) gene family. SCGB2A2 has previously been found overexpressed in breast tumors but possible associations between its expression and established prognostic tumor characteristics such as the levels of estrogen and progesterone receptors have not yet been investigated. We evaluated SCGB2A2 expression at the mRNA and at the protein level by reverse-transcription polymerase chain reaction and immunocytochemistry in 52 and 32 breast tumors, respectively. Both SCGB2A2 mRNA and protein expression were significantly higher in estrogen-receptor-positive compared to estrogen-receptor-negative tumors (Mann–Whitney rank sum test, $p = 0.04$; chi-square test, $p = 0.01$; respectively). In contrast, SCGB2A2 expression did not correlate with progesterone receptor levels or Notthingham grade. As estrogen and antiestrogen treatment of estrogen-positive breast cancer cell lines does not modify SCGB2A2 expression we suggest that SCGB2A2 may be a new independent breast cancer prognostic marker.

Key Words: SCGB2A2; MGB1; estrogen receptor; progesterone receptor; Notthingham grade; breast cancer.

Introduction

Mammaglobin (MGB1, SCGB2A2) was first identified in 1996, using differential display analysis, as a breast-specific member of the secretoglobin (SCGB) gene family overexpressed in some breast tumors *(1,2)*. Today, a search for breast-specific expressed sequence tags (ESTs) performed using the Differential Gene Expression Displayer (DGED) tool at the Cancer Gene Anatomy Project (CGAP) website (http://cgap.nci.nih.gov/Tissues/GXS) shows that SCGB2A2-related ESTs have been found in nine different breast cDNA libraries but only two non-breast libraries, further confirming the relative breast specificity of SCGB2A2 expression. Using a subtractive hybridization approach, we previously identified SCGB2A2 mRNA as overexpressed in the *in situ* compared to the invasive element within an individual breast tumor *(3,4)*. Further *in situ* hybridization analysis, performed in breast tumors selected to include normal, *in situ*, and invasive primary tumor elements revealed that SCGB2A2 expression, restricted to epithelial cells, could be detected in all elements and was significantly increased in tumor cells compared to normal cells *(4)*. This higher SCGB2A2 expression in malignant versus nonmalignant breast epithelium has also been confirmed at the protein level by immunocytochemistry *(5)*. In this latter study, Watson et al. concluded that SCGB2A2 expression was independent of tumor grade and histological type.

It has recently been demonstrated that circulating mammary carcinoma cells can also be detected in the blood of breast cancer patients via PCR detection of SCGB2A2 mRNA *(6–9)*. Even though its biological function remains unknown, SCGB2A2 is now considered as a relatively specific marker of axillary lymph node breast metastases as well as of occult breast cancer *(10–13)*. Interestingly, Zach et al. detected SCGB2A2 mRNA expression by nested reverse-transcription PCR (RT-PCR) more frequently in the blood of patients with estrogen-receptor-positive (ER+) breast tumor than in the blood of estrogen-receptor-negative (ER–) breast cancer patients, suggesting a possible relationship between SCGB2A2 and ER levels in primary breast tumors *(6)*. In order to investigate further possible associations between SCGB2A2 expression and estrogen and progesterone receptors in primary breast tumors, we assessed SCGB2A2 expression at the mRNA and at the protein level in a cohort of breast tumors.

Results

Assessment of SCGB2A2 mRNA Expression in a Cohort of 52 Human Breast Tumor Samples

To establish whether SCGB2A2 mRNA expression paralleled established known prognostic parameters such as ER and PR levels, a cohort of 52 cases was selected from the

Received May 20, 2003; Revised May 29, 2003; Accepted May 30, 2003.
Author to whom all correspondence and reprint requests should be addressed: Etienne Leygue, Department of Biochemistry and Molecular Biology, University of Manitoba, 770, Bannatyne Avenue, Winnipeg, MB. R3EOW3, Canada.

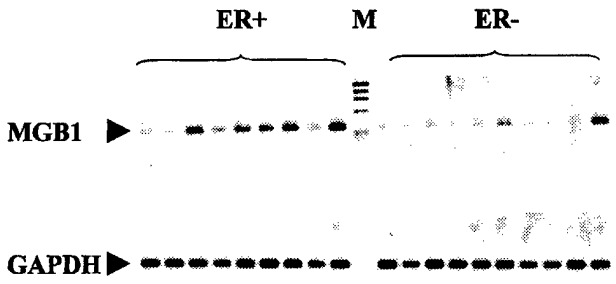

Fig. 1. RT-PCR analysis of SCGB2A2 and GAPDH mRNA expression in primary breast tumors. Total RNA was extracted from frozen tissue sections corresponding to ER positive (ER+) and ER negative (ER–) cases, reverse-transcribed and PCR amplified as described in the Materials and Methods section using SCGB2A2- or GAPDH-specific primers. PCR products were then separated on 2% agarose gels prestained with ethidium bromide. Black arrow: product corresponding to SCGB2A2, grey arrow: product corresponding to GAPDH. M: Molecular weight marker (Φx174 RF DNA/*Hae*III fragments, Gibco BRL, Grand Island, NY).

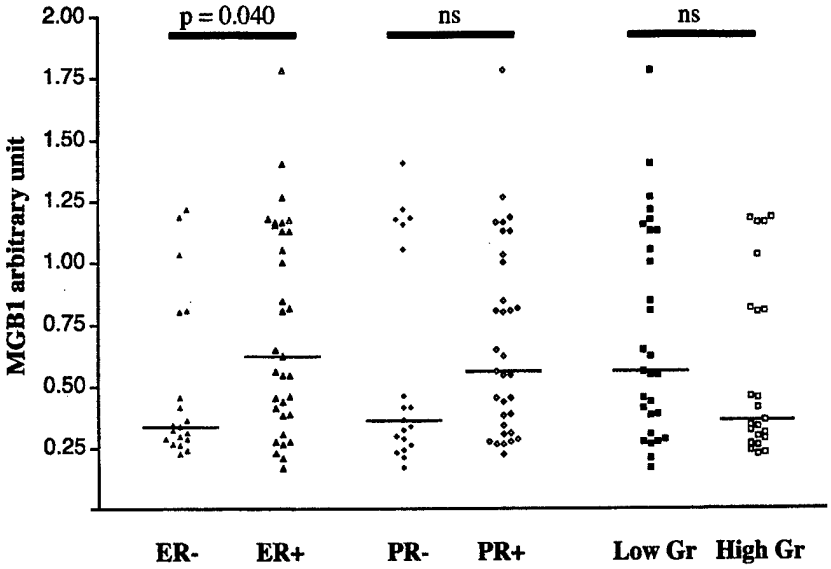

Fig. 2. Quantification of SCGB2A2 mRNA expression in different breast tumor subgroups. Total RNA was extracted from frozen tissue sections corresponding to 52 cases and analyzed as described in Fig. 1. SCGB2A2 mRNA expression was quantified relative to GAPDH mRNA as described in the Materials and Methods section. Tumors were grouped according to their ER status (ER+, ER–), their PR status (PR+, PR–) or their grade (Low Gr: Notthingham scores between 5 and 7; High Gr: Notthingham scores between 8 and 9). Difference between subgroups were tested using the Mann–Whitney rank sum test, two-sided.

NCIC-Manitoba Breast Tumor Bank. For each case, clinical characteristics of the tumor (i.e., ER and PR levels, Nottingham grade) were known (*see* Materials and Methods for a summary of tumor subgroup characteristics). Total RNA was extracted from frozen primary tumor sections, reverse-transcribed and analyzed by RT-PCR using primers recognizing specifically SCGB2A2 cDNA, and chosen to span intronic regions. As shown Fig. 1, SCGB2A2 corresponding signal can be detected in the majority of cases, even though levels of expression varied from one sample to another. Amplification of the ubiquitously expressed GAPDH cDNA in the same cDNA samples was performed in parallel and, for each case, a normalized SCGB2A2 mRNA expression value was calculated (*see* Materials and Methods). SCGB2A2 expression was found to strongly correlate with ER levels ($n = 52$, Spearmann coefficient $r = 0.282$, $p = 0.042$) but not with PR levels or grade (data not shown). Similarly (Fig. 2), using the established clinical cut-off of ER positivity (ER positive tumors have a binding higher than 3 fmol/mg of total protein), SCGB2A2 mRNA expression was significantly (Mann–Whitney rank sum test, two-sided, $p = 0.040$) higher in ER+ ($n = 33$, median value SCGB2A2 = 0.62) than in ER– ($n = 19$, median SCGB2A2 value = 0.33).

Assessment of SCGB2A2 Protein Expression in a Cohort of 32 Human Breast Tumor Samples

In order to determine whether SCGB2A2 protein expression correlated with SCGB2A2 mRNA expression and whether a similar association between ER status and SCGB2A2 expression could be observed at the protein level, paraffin

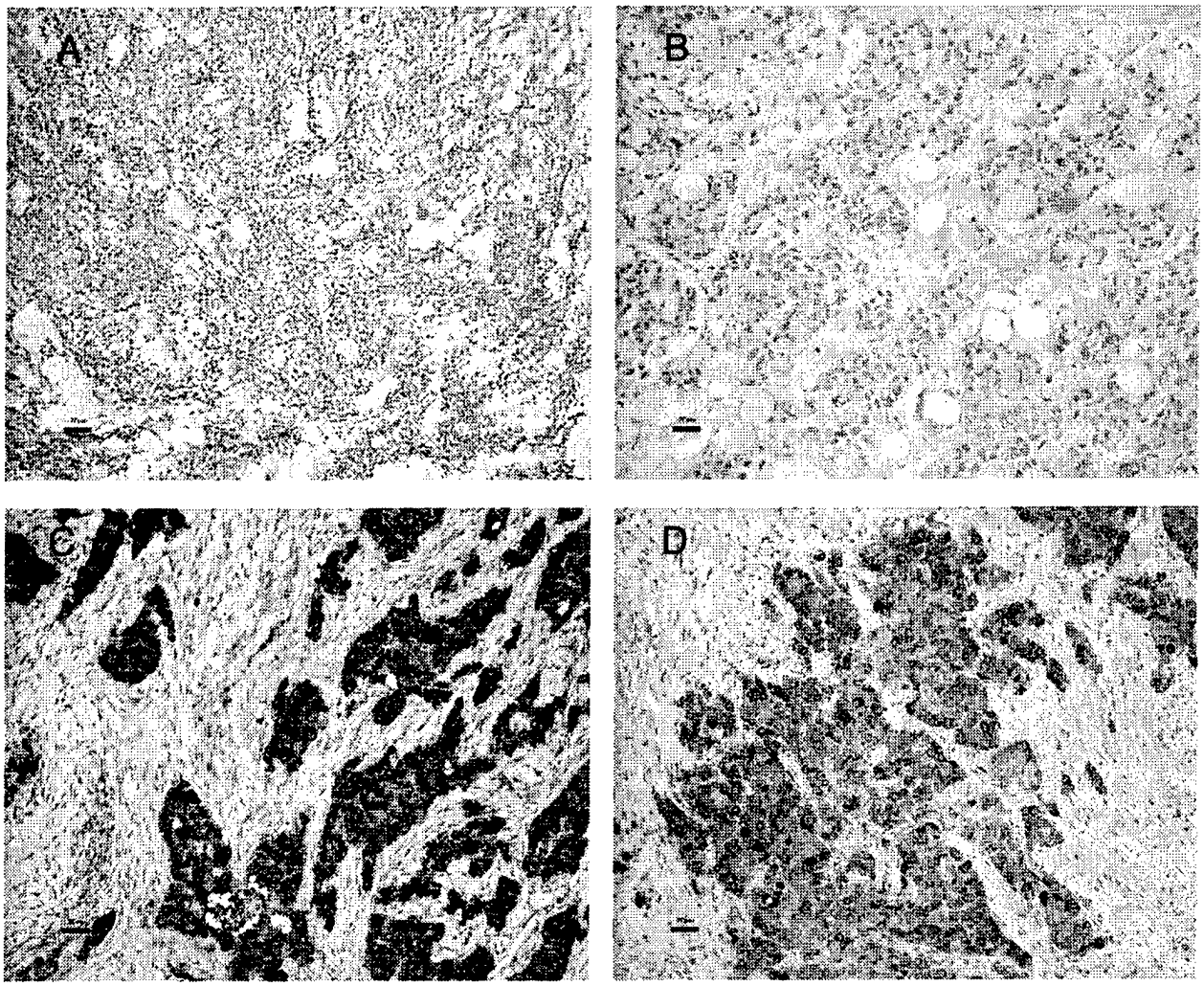

Fig. 3. Detection of SCGB2A2 protein in breast tumors by immunohistochemistry. SCGB2A2 protein was detected on paraffin-embedded breast tumor tissue sections using a rabbit polyclonal primary anti-SCGB2A2 antibody (Kindly provided by Dr. T Fleming) and the Ventana-Discovery system as described in the Materials and Methods section. Panel A and B: Two independent ER– cases showing no (A) or low (B) SCGB2A2 protein expression in tumor epithelial cells. Panel C and D: two independent ER+ cases presenting a strong SCGB2A2 signal. Blue bar: 20 μm.

blocks corresponding to 32 out of these 52 tumors were sectioned and processed for immunohistochemical analysis of SCGB2A2 expression (*see* Materials and Methods). Slides were scored blindly for SCGB2A2 protein expression by a pathologist as described in the Materials and Methods section. Some sections showed no (Fig. 3A, SCGB2A2 score = 0) or low (Fig. 3B, SCGB2A2 score = 1) SCGB2A2 expression, whereas others presented strong SCGB2A2 protein signal (Fig. 3C, SCGB2A2 score = 3; Fig. 3D, SCGB2A2 score = 2). Comparison of SCGB2A2 protein scores and previously obtained normalized SCGB2A2 mRNA levels revealed a strong correlation ($n = 32$, Spearman r coefficient $r = 0.575$, $p = 0.0006$) between protein and mRNA levels. Tumors were classified as low (scores between 0 and 1) and high (1.5 and 3) SCGB2A2 protein expressors, and differences between tumor subgroups (ER+/ER–, PR+/PR–, low grade/high grade) were assessed using chi-square test. As observed for SCGB2A2 mRNA, SCGB2A2 protein positivity was associated (chi-square test, $p = 0.017$) with ER status but not with PR status or grade (Fig. 4).

Absence of Estrogen Regulation of SCGB2A2 Expression

These data suggested that estrogen might regulate SCGB2A2 expression. In order to address the question of a possible regulation of SCGB2A2 expression in breast cancer cells, ZR-75 cells, known to express SCGB2A2 *(14)*, were treated by estradiol-17β 10^{-8} *M* or the antiestrogen ICI-182,780 10^{-6} *M* for 6, 24, and 48 h as described in the Materials and Methods section. Total RNA was extracted and analyzed by RT-PCR using primers recognizing GAPDH, SCGB2A2, or psoria-

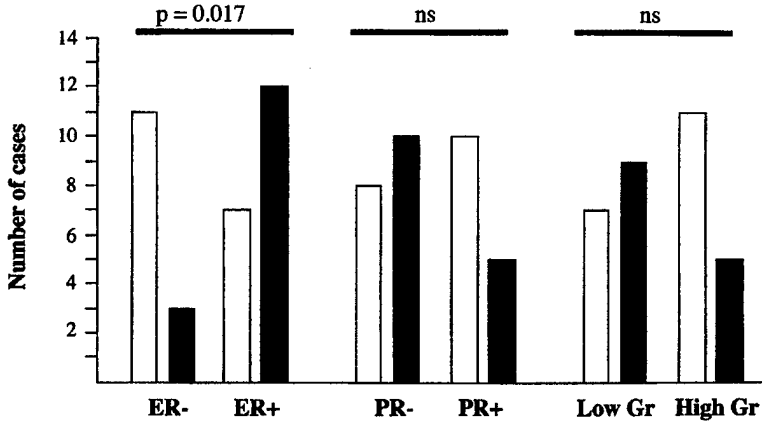

Fig. 4. Quantification of SCGB2A2 protein in breast tumor subgroups. Paraffin-embedded tissue section corresponding to 32 cases were processed as shown Fig. 3. Slides were independently reviewed and scored as described in the Materials and Methods section. For each tumor subgroup (ER–, ER+, PR+, PR–, low grade, and high grade), the number of cases negative (White columns) or positive (black columns) is shown. Differences between subgroups were tested using the chi-square test.

sin cDNAs. Psoriasin was chosen as its expression has previously been shown to be regulated by estrogen treatment (*15*, our unpublished data). SCGB2A2 mRNA expression was not changed under any treatment condition (data not shown), whereas, as expected, psoriasin signal was found to be increased by estradiol and decreased by antiestrogen treatment as soon as 6 h of treatment, with a maximum effect after 24 and 48 h of treatment (estradiol treatment: 1.5-, 2.8-, and 4.5-fold control and antiestrogen treatment 0.90-, 0.80-, and 0.70-fold control, respectively).

Discussion

Assessment of SCGB2A2 expression at the mRNA and the protein levels in a cohort of breast tissue samples showed a statistically significant relationship between SCGB2A2 levels and ER status. However, within the same cohort, no association was found between SCGB2A2 expression and other known prognostic marker such as PR levels or Notthingham grade.

To the clinician, a factor is considered a prognostic factor when it is associated with the outcome of the disease, i.e., predicts how the disease would evolve if not treated, whereas a predictive factor is associated to the degree of response to therapy, i.e., predicts the likelihood of response to a particular treatment. A high level of ER in tumor tissue has a good prognostic value and also predicts a good likelihood of responding to hormonal adjuvant therapy such as tamoxifen *(16,17)*. As PR expression is positively regulated by estrogens, higher PR levels in ER+ tumors support the hypothesis of an operational ER signaling pathway and is therefore also considered as a good prognostic and predictive parameter. Whereas the parallel between SCGB2A2 and ER expression suggested that SCGB2A2 could be a new ER target gene, the lack of association with a known regulated gene such as PR suggested that SCGB2A2 expression was independent of ER signaling pathway. This latter hypothesis was further supported by the absence of estrogen and antiestrogen regulation of SCGB2A2 expression in ZR-75 cells, even though ER signaling pathway appears functional, as shown by the induction of a known ER-regulated gene, psoriasin. It should be noted that a similar absence of regulation was also observed in another ER+ breast cancer cell line MCF-7 cells (our unpublished results; *18*). However, even though the SCGB2A2 gene was not grossly rearranged in MCF-7 cells *(18)*, these cells do not express endogenous SCGB2A2 (our unpublished results; 2). It might therefore be hypothesized that SCGB2A2 expression in MCF-7 cells is negatively regulated by other factors, resulting in an absence of estrogen regulation in these cells. Further experiments performed on other breast cancer cell lines and primary cells *(19)* are needed to confirm these preliminary results.

Interestingly, the general expression of SCGB2A2 as well its association with ER levels observed in vivo in breast tissue contrasts with in vitro observations made on mammary epithelial cancer cell lines. Indeed, looking at a panel of different breast cancer cell lines, Watson et al. reported the detection of SCGB2A2 transcripts only in few cell lines (MB361, MB415, MB468, BT474, MB175) with no expression in MCF7, MB134, MB231, or MCF10A cells *(2)*. Similarly, we did not detect SCGB2A2 expression in breast cell lines such as BT20, T47D, or MCF10AT1 even though a strong signal was seen in ZR-75 (our unpublished observation). As cells such as MB468 and MB361 are ER– and cells such as ZR-75 or BT474 are ER+, SCGB2A2 expression does not appear related to ER status in cells grown in vitro. Overall, this suggests that most of cell lines, through

selection, medium conditions, and/or dedifferentiation lost their ability to express SCGB2A2 in vitro. Presently, no data are available regarding the possible biological function of SCGB2A2. It has however recently been reported that SCGB2A2 existed in a tetrameric complex with BU101 (lipophilin B), another member of the secretoglobin family, the expression of which correlated with SCGB2A2 expression in breast tissue *(20)*. The role of this complex as well as the possible regulation of its components remains to be determined.

In conclusion, we found that SCGB2A2 expression correlated with ER levels in breast tumor tissue. As ER is considered as a good prognostic factor and as SCGB2A2 does not appear to be directly regulated by the ER signaling pathway, we hypothesize that SCGB2A2 expression may be a new independent prognostic marker in breast cancer. Further experiments performed on a larger cohort of patients and completed with follow up studies are needed to test this hypothesis.

Materials and Methods

Human Breast Tissues and Cell Lines

All breast tumor cases used for this study were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As it has been previously described *(21)*, tissues are accrued to the Bank from cases at multiple centers within Manitoba, rapidly collected and processed to create matched formalin-fixed embedded and frozen tissue blocks for each case with the mirror image surfaces oriented by colored inks. The histology of every sample in the Bank is uniformly interpreted by a pathologist in hematoxylin and eosin (H&E)-stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific paraffin and frozen blocks. Fifty two tumors were selected, spanning a wide range of estrogen and progesterone receptor levels, as determined by ligand binding assay. Within these tumors, 9 were ER–/PR– (ER < 3 fmol/mg total protein; PR < 10 fmol/mg), 10 were ER–/PR+ (ER < 3 fmol/mg; PR > 10 fmol/mg), 10 were ER+/PR– (ER > 3 fmol/mg; PR < 10 fmol/mg), and 23 were ER+/PR+ (ER > 3 fmol/mg, PR > 10 fmol/mg). These tumors also spanned a wide range of Nottingham grade for ER– (n = 19, grade ranging from 5 to 9, median 8) and ER+ (n = 33, grade ranging from 5 to 9, median 6) tumors. SCGB2A2 mRNA expression was assessed by RT-PCR on total RNA extracted from frozen tissue sections. Paraffin blocks corresponding to 32 out of these 52 tumors were sectioned and processed for immunohistochemical analysis of SCGB2A2 expression.

ZR-75 cells, ER+ breast cancer cells known to express SCGB2A2, were grown and treated with estradiol-17β 10^{-8} M in charcoal-stripped medium or with the antiestrogen ICI 182,780 (10^{-6} M) in regular medium for 6, 24, or 48 h, as previously described *(22)*. Total RNA was extracted from frozen tissue sections or cell lines using Tri-reagent (MRCI, Cincinnati, OH).

RT-PCR Analysis

One microgram of total RNA was reverse transcribed in a final volume of 20 μL and 1 μL of the reaction mixture subsequently amplified by PCR as previously described *(23, 24)*. Primers used corresponded to SCGB2A2 (sense 5'-CCGACAGCAGCAGCCTCAC-3', located in SCGB2A2 sequence between bases 41 and 59, and antisense 5'-TCCG TAGTTGGTTTCTCAC-3', located between bases 401 and 383) *(2)*; to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (sense 5'-ACCCACTCCTCCACCT TTG-3' and antisense 5'-CTCTTGTGCTCTTGCTGGG-3'); and to psoriasin *(24)* gene (sense 5'-AAGAAAGATGA GCAACAC-3' and antisense 5'-CCAGCAAGGACAGA AACT-3'). To amplify cDNA corresponding to SCGB2A2, GAPDH, and psoriasin, 30 cycles (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C) of PCR were used. Ten microliters of PCR products were loaded on prestained (15 μg/mL ethidium bromide) 2% agarose gels. Identity of fragments corresponding to SCGB2A2, GAPDH, and psoriasin had previously been confirmed by sequencing.

Three independent PCRs were performed using SCGB2A2, psoriasin, and GAPDH primers and signals, visualized with UV irradiation on a GelDoc2000/ChemiDoc System (Biorad), were quantified by densitometry using the Quantity One software (Version 4.2, Biorad). SCGB2A2 and psoriasin expression were expressed relative to GAPDH expression as previously described *(25)*. Briefly, three independent PCRs were performed using each set of primers. In order to control for variations between experiments, a value of 1 was arbitrarily assigned to the signal of one particular tumor measured in each set of PCR experiments (always the same tumor) and all signals were expressed relative to this signal. Levels of SCGB2A2 were then expressed relative to the GAPDH signal corresponding to each individual tumor sample. Correlation between normalized SCGB2A2 expression and tumor characteristics was tested by calculation of the Spearman coefficient, r. Comparison between tumor subgroups was performed using the Mann–Whitney rank sum test, two-sided.

Immunohistochemical Analysis of SCGB2A2 Expression

Detection of SCGB2A2 protein was performed using an antibody previously characterized and kindly provided by Dr. Timothy Fleming *(1,2,5,18)*. Paraffin-embedded breast tissue sections were processed using the automated Discovery Staining Module, Ventana System (Tucson, Arizona) and the Research IHC DAB paraffin protocol according to the manufacturer's instructions. All steps were performed automatically: briefly, following deparaffination of tissue

sections, slides were incubated 60 min at 42°C in the presence of rabbit anti-SCGB2A2 antibody (1/1000 final concentration), washed, incubated with biotinylated secondary anti-rabbit antibody (14 minutes 42°C), washed, incubated 8 min with avidin–HRPO complex subsequently detected with DAB-H_2O_2 solution. Counterstaining was also performed automatically by the Ventana apparatus (hematoxylin/bluing reagent).

Levels of mammaglobin expression were assessed by bright-field microscopic examination at low-power magnification and using a previously described semiquantitative approach *(25)*. Scores were obtained by estimating average signal intensity (on a scale of 0 to 3) and the proportion of epithelial cells showing a positive signal (0, none; 0.1, less than one-tenth; 0.5, less than one-half; 1.0 greater than one-half). The intensity and proportion scores were then multiplied to give an overall score. Cases with a score lower than or equal to 1 were considered negative or weakly positive, whereas tumors with scores higher than 1.0 were classified as positive for SCGB2A2 expression. Statistical comparisons between tumor subgroups have been performed using the chi-square test.

Acknowledgments

This work was supported by grants from the Manitoba Health research Council (MHRC) and the U.S. Army Medical Research and Materiel Command (USAMRMC). P. H. W. is a Medical Research Council of Canada (MRC) Clinician-Scientist, E. L. is a recipient of a USAMRMC Career Development Award (CDA BC000577).

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Appendix 2 Cancer Res. 2002. 62: 2736-2740

[CANCER RESEARCH 62, 2736–2740, May 15, 2002]

Advances in Brief

Identification of a Novel Breast- and Salivary Gland-specific, Mucin-like Gene Strongly Expressed in Normal and Tumor Human Mammary Epithelium[1]

Richard J. Miksicek,[2,3] Yvonne Myal,[2] Peter H. Watson, Christina Walker, Leigh C. Murphy, and Etienne Leygue

Department of Physiology, Michigan State University, East Lansing, Michigan 48824 [R. J. M.]; Department of Pathology, University of Manitoba, Winnipeg, Manitoba, R3E 0W3 Canada [Y. M., P. H. W.]; Department of Biochemistry and Medical Genetics and Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Manitoba, R3E 0W3 Canada [P. H. W., C. W., L. C. M., E. L.]

Abstract

Expression profiling using the public expressed sequence tag (EST) and serial analysis of gene expression (SAGE) databases resulted in the identification of a putative breast-specific mRNA that we have termed small breast epithelial mucin (SBEM). Hybridization analysis performed on 43 normal human tissues revealed that the *SBEM* gene was only expressed in mammary and salivary glands. Further reverse-transcription PCR analyses confirmed SBEM expression in most of established human breast epithelial cell lines analyzed (7 of 8) but not in cell lines of non-breast origin (0 of 6). *SBEM* mRNA expression was detected in >90% of invasive ductal carcinomas and correlated with the expression of a previously characterized breast-specific gene, mammaglobin-1 ($n = 54$; Spearman $r = 0.34$, $P = 0.011$). Interestingly, a higher SBEM:mammaglobin-1 ratio was observed in primary tumors with axillary lymph node metastasis than in node-negative tumors ($n = 46$; Mann-Whitney, $P = 0.04$). In a subset of 20 primary breast tumors and their matched axillary lymph nodes, a high concordance (Fisher's exact test, $P < 0.001$) was seen between PCR detection of *SBEM* mRNA in lymph node tissue and their histopathological status, indicating that SBEM mRNA expression is conserved in nodal metastasis. The *SBEM* gene is predicted to code for a putative low molecular weight, secreted sialoglycoprotein, potentially useful for the diagnosis of metastatic breast cancer.

Introduction

Early detection remains a central goal in breast cancer treatment to enable intervention at a localized and potentially curable stage and to maximize the opportunity for breast conservation. The 5-year survival rate for women with breast cancer increases dramatically when it can be diagnosed at an early stage, from >95% in patients with a localized tumor to ~75% with regional disease and <25% in women with disseminated cancer (1). Nevertheless, only 60% of all breast cancers are diagnosed at a local stage, and any improvement in early detection would have a significant impact on reducing overall breast cancer mortality.

Improving the diagnosis and clinical management of breast cancer requires access to a wider range of biomarkers able to reflect the molecular phenotype of breast tissue. A special need exists to identify novel genes whose expression is restricted to the mammary epithelium, because these genes have the greatest potential to enhance detection of micrometastatic disease and the potential to report on proliferative changes in the breast, analogous to the ability of elevated serum prostate-specific antigen levels to indicate the presence of hyperplasia or cancer of the prostate gland (2).

The identification of new tissue-specific markers has benefited especially from expansion of public and private databases for ESTs[4] (3, 4) and by large-scale efforts to profile patterns of gene expression using techniques such as serial analysis of gene expression (5). Using sequence analysis software and web-based tools developed for molecular profiling, we have identified a novel putative breast-specific gene, belonging to a recently regrouped cluster (UniGene identifier Hs.348419),[5] which represents an attractive candidate for a breast tumor marker with obvious potential for cancer diagnostics.

Materials and Methods

Database and Sequence Analysis. The cDNA xProfiler tool[6] was used to search for novel breast-specific ESTs. Protein sequence analysis used the SignalP algorithm[7] to search for the presence of a signal sequence (6) and the NetOGlyc algorithm[8] to predict sites of potential glycosylation (7).

RNA Hybridization Analysis for Tissue Specificity. A ^{32}P-labeled SBEM probe, generated using the cloned SBEM PCR product (396 bp) and the RadPrime DNA labeling system (Life Technologies, Inc., Burlington, Ontario, Canada), was hybridized to a commercially available RNA Master Blot (Clontech, Palo Alto, CA), containing poly(A)+ RNA (100–500 ng) isolated from a variety of adult and fetal human tissues, according to the manufacturer's instructions.

Cell Culture and RNA Preparation. Cell lines were obtained from the American Type Culture Collection or other sources and were cultured as follows: DMEM with 10% fetal bovine serum (MCF7, MCF10AT1, MCF10AT3c, SK-UT-1B, and HepG2); DMEM with 10% calf serum (MDA MB-231, Hec 1A, and HeLa); DMEM:Ham's F12 (1:1) with 10% fetal bovine serum (ZR-75-1 and RL95-2); RPMI 1640 with 10% fetal bovine serum (T-47D and LNCaP); or MSU-1 medium (8) with 5% fetal bovine serum (M13SV-1). All media were supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), HEPES (pH 7.4; 5 mM), and glutamine (2 mM). MCF7, T-47D, and ZR-75-1 cells also received bovine insulin (10 μg/ml). Media and sera were obtained from Life Technologies, Inc. (Life Technologies, Inc., Grand Island, NY). RNA was extracted from cultured cells using guanidinium isothiocyanate, followed by centrifugation through a 5.7 M cesium chloride cushion as described (9). RNA from cultured primary HMECs obtained by reduction mammoplasty was a kind gift from P. Ervin (Biotherapies, Inc., Ann Arbor, MI).

Breast Tumors and Axillary Lymph Nodes. Fifty-four invasive ductal carcinomas were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). Cases spanned many ER (0–298 fmol/mg protein) and PR (0–1199 fmol/mg protein) levels, as determined by ligand binding assay. Tumors also spanned many grades (Nottingham grade scores from 5 to 9). For

Received 11/8/01; accepted 3/28/02.

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[1] Supported by R21 CA094943-01 (to R. J. M.), NSERC (to Y. M.), and BC000577 (to E. L.).

[2] These authors contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Department of Physiology, Michigan State University, East Lansing, MI 48824-1101. Phone: (517) 355-6475, extension 1285; Fax: (517) 355-5125; E-mail: miksicek@msu.edu.

[4] The abbreviations used are: EST, expressed sequence tag; SBEM, small breast epithelial mucin; HMEC, human mammary epithelial cell; ER, estrogen receptor-α; PR, progesterone receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde phosphate dehydrogenase; MUC1, mucin 1.

[5] Internet address: http://www.ncbi.nlm.nih.gov/UniGene/. UniGene is a system for automatically partitioning GenBank sequences, including ESTs, into a nonredundant set of gene-oriented clusters.

[6] Internet address: http://cgap.nci.nih.gov/CGAP/Tissues/xProfiler.

[7] Internet address: http://www.cbs.dtu.dk/services/SignalP/.

[8] Internet address: http://www.cbs.dtu.dk/services/NetOGlyc/.

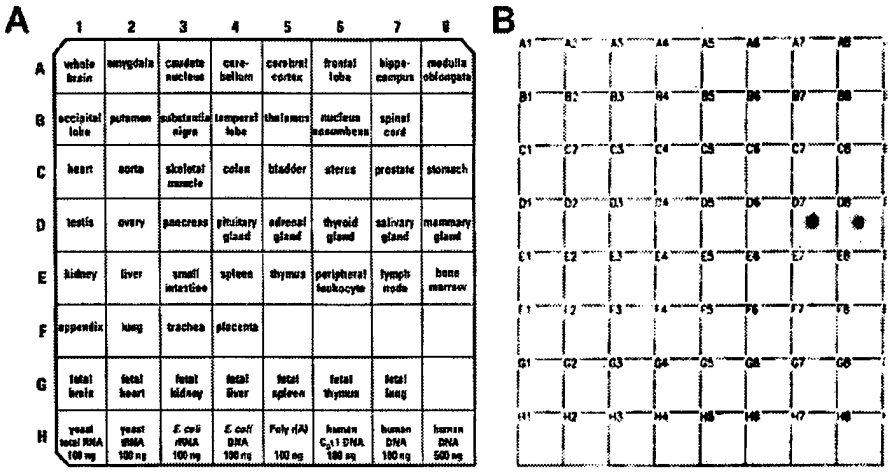

Fig. 1. Tissue expression of *SBEM* mRNA. An RNA Master Blot (Clontech Laboratories, Palo Alto, CA) containing poly(A)+ RNAs from different human tissues (*A*) was screened with ^{32}P-labeled SBEM probe as described in "Materials and Methods." The SBEM transcript (*B*) was expressed in the human salivary (*D7*) and mammary gland (*D8*).

46 tumors, pathological axillary lymph node status (presence or absence of metastasis) was known.

In a subset of cases (n = 20), frozen primary human breast tumor samples and their matched frozen lymph nodes containing (n = 14) or not (n = 6) histologically detectable metastatic cancer cells were available from the Manitoba Breast Tumor Bank. For the primary tumor samples, the ER levels, determined by ligand binding assays, ranged from 2.3 fmol/mg protein to 298 fmol/mg protein, whereas PR levels ranged from 10.1 fmol/mg protein to 112 fmol/mg protein.

RNA Analysis by RT-PCR. Total RNA was extracted from 20-μm frozen tissue sections (five sections/tumor) and reverse transcribed as described previously (10). The primers used for SBEM amplification consisted of SBEM-U (5′-CTTTGAAGCATTTTTGTCTGTG-3′; sense) and SBEM-L (5′-AAGGTAAGTAGTTGGATGAAAT-3′; antisense). PCR amplifications were performed, and PCR products were analyzed as described previously (11), with minor modifications. Briefly, aliquots of each reverse transcription mixture (2 μl for Fig. 2 and 0.8 μl for Fig. 3) were amplified in a final volume of 20 μl, in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 5 ng/μl of each SBEM primer, and 0.5 unit of Taq DNA polymerase. Each PCR consisted of 35 cycles (15 s at 94°C, 30 s at 58°C, and 60 s at 74°C).

Primers used for mammaglobin-1 were: Mam-1 (5′-CCGACAGCAG-CAGCCTCAC-3′, sense strand) and Mam-2 (5′-TCCGTAGTTGGTTTCT-CAC-3′, antisense strand). Primers for the ubiquitously expressed *GAPDH* gene were GAP-1 (5′-ACCCACTCCTCCACCTTTG-3′, sense strand) and GAP-2 (5′-CTCTTGTGCTCTTGCTGGG-3′, antisense strand). To amplify cDNA corresponding to mammaglobin-1 and GAPDH, 30 cycles of PCR were used (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C). All buffers were the same as for SBEM PCR, except that 2 mM $MgCl_2$ was used when amplifying mammaglobin-1 cDNA. PCR products were then separated on a 1.5% agarose gel. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/ml).

Quantification and Statistical Analysis. Three independent PCRs were performed for tumor specimens using SBEM, mammaglobin-1, and GAPDH primers and signals, visualized with UV irradiation on a GelDoc2000/Chemi-Doc System (Bio-Rad), were quantified by densitometry using the Quantity One software (version 4.2; Bio-Rad). SBEM and mammaglobin-1 expression was normalized to GAPDH expression as described previously (11). Correlation between SBEM expression and tumor characteristics or mammaglobin-1 expression was tested by calculation of the Spearman coefficient r. Differences between tumor subgroups were tested using the Mann-Whitney two-tailed test or Fisher's exact test.

***In Situ* Analysis of SBEM mRNA Expression.** *In situ* hybridization was performed on adjacent paraffin-embedded breast tumor tissue sections corresponding to a case shown to express high levels of *SBEM* mRNA by RT-PCR, using SBEM ^{32}P-labeled sense and antisense probes, as described previously (12).

Results

Identification of a Putative Novel Breast-specific Gene. The cDNA xProfiler tool (see "Materials and Methods") was used to identify tissue-restricted cDNAs with preferential representation in libraries prepared from normal breast tissue and breast tumors. This search identified a new cluster of ESTs now grouped under the UniGene identifier number Hs.348419. Of the 30 ESTs found in this cluster, 15 are ascribed to breast cDNA libraries, 9 were isolated from random activation of gene expression or pooled tissues, 5 were isolated from fetal sources (fetal heart and fetal skin), and 1 came from a head and neck tumor cDNA library. Alignment of these ESTs led to the construction of a 500-bp consensus cDNA sequence containing a 90-amino acid open reading frame in which the initiating methionine is framed by a nearly perfect consensus motif for translation initiation (5′-CCACCATGA-3′; Ref. 13). Further database analysis showed that this sequence, interrupted by three introns, is present on chromosome 12q13.2. Primers were designed to span the open reading frame, and we cloned a 396-bp fragment from both MCF-7 cells and breast tissue, which we called SBEM (GenBank accession number AF414087). The presence of a hydrophobic signal peptide (residues 1–19; Ref. 6) within the protein sequence (GenBank accession number AAL02119) suggests that SBEM is a secreted protein subject to proteolytic processing. The NetOGlyc glycosylation algorithm (7) further predicts this protein to be *O*-glycosylated on most of its 16 threonine residues. The SBEM protein contains three tandem copies of a neutral octapeptide core repeat (ThrThrAlaAlaXxxThrThrAla, where Xxx corresponds to Ala, Pro, or Ser). The NH_2 and COOH termini of the processed polypeptide are otherwise charged and fairly polar. These features suggest strong similarity to many sialomucins, although this protein lacks a transmembrane domain and is substantially shorter than most other known epithelial mucins (14, 15).

Expression of *SBEM* mRNA Is Restricted to the Mammary and Salivary Glands. Database searches suggested that SBEM expression was mainly restricted to breast tissue. To confirm this prediction, we performed hybridization analysis with an RNA MasterBlot containing highly purified polyadenylated RNA from 43 adult and 7 fetal human tissues arrayed on a nylon membrane. A *SBEM* cDNA probe hybridized exclusively to mRNA from the mammary and salivary glands (Fig. 1). Of note, no expression was observed in colon, lung, uterus, ovary, liver, pancreas, kidney, or prostate, all of which represent common primary tumor sites. Additionally, no hybridization to any of the fetal RNAs was observed.

***SBEM* mRNA Is Expressed in Breast Cancer Cell Lines but not in Cell Lines of Non-Breast Origin.** The profile of *SBEM* mRNA expression was further assessed using RT-PCR, followed by PCR amplification, in a panel of human breast and non-breast cell lines. A SBEM PCR product of the expected size (396 bp) was readily detected in MCF7 and ZR-75-1 breast tumor cells (data not shown). Lower but reproducible expression was also observed in primary HMECs and in several established breast epithelial cell lines including T-47D, M13SV-1 (8), MCF10AT1, and MCF10AT3c (16). MDA MB-231 breast tumor cells were negative for SBEM expression, as were six tumor cell lines of non-breast origin (uterus: RL95-2, SK-UT-1B, Hec 1A; cervix: HeLa; prostate: LNCaP; and liver: HepG2). As controls, we also examined the expression of a housekeeping gene (*GAPDH*) and mammaglobin-1, an established mammary-specific gene that is being independently investigated as a promising marker for breast tumor diagnosis and nodal metastasis (12, 17). Of the cell lines tested, only HMEC and ZR-75-1 cells expressed mammaglobin-1, consistent with published reports.

Analysis of *SBEM* mRNA in Human Breast Tumors. Northern blot analyses performed on a small series of 10 cases revealed that *SBEM* mRNA was 600 bp long and differentially expressed from one sample to another (data not shown). To determine whether *SBEM* mRNA was widely expressed in human breast tumor tissue, 54 human breast tumors, spanning many ER and PR levels as well as tumor grade and nodal status, were selected from the Manitoba Breast Tumor Bank. Total RNA was extracted from frozen tissue sections and reverse transcribed. PCR amplification of *GAPDH* (control), *mammaglobin-1,* and *SBEM* cDNA was then performed. A PCR product, 396-bp long was detected in all but three tumors (data not shown) when using SBEM-specific primers. After cloning and sequencing, this product was shown to correspond to *SBEM* cDNA. Quantification of the *SBEM* signal relative to the *GAPDH* signal was performed as described in "Materials and Methods." No correlation was found between SBEM expression and tumor characteristics such as ER ($n = 54$; Spearman $r = -0.01$, $P = 0.89$) and PR ($n = 54$; Spearman $r = -0.03$, $P = 0.77$) levels or tumor grade ($n = 44$; Spearman $r = -0.06$, $P = 0.68$). Interestingly, however, the *SBEM* signal correlated positively with *mammaglobin-1* expression ($n = 54$; Spearman $r = 0.340$, $P = 0.011$). Subgroup comparison of *SBEM* and *mammaglobin-1* expression confirmed our previous observation[9] that *mammaglobin-1* expression is higher in ER-positive and low-grade tumors (Table 1). Interestingly, although not statistically significant ($P = 0.09$), higher SBEM expression was found in lymph node-positive compared with node-negative tumors. Also of interest is the fact that the SBEM:mammaglobin-1 ratio is significantly ($n = 46$; Mann-Whitney, $P = 0.04$) higher in these lymph node-positive tumors.

[9] E. Leygue, L. C. Murphy, and P. H. Watson, unpublished results.

Table 1 *Median values (arbitrary units) of SBEM expression, mammaglobin-1 (Mam) expression, and SBEM:mammaglobin-1 ratio in different tumor subgroups*

Tumors	*n*	SBEM	*P*	Mam	*P*[a]	SBEM: Mam	*P*[a]
ER +	34	2.29	0.66	0.63	**0.04**	3.39	0.22
ER −	20	2.15		0.34		4.44	
PR +	34	2.30	0.63	0.59	0.27	3.56	0.50
PR −	20	1.97		0.38		3.58	
Node +	36	2.30	0.09	0.45	0.62	3.97	**0.04**
Node −	10	1.96		0.70		2.42	
Grades 5–7	24	2.05	0.84	0.59	**0.03**	3.33	0.10
Grades 8–9	20	2.24		0.34		4.02	

ER +, >3 fmol/mg of protein; ER −, ≤3 fmol/mg of protein; PR +, >10 fmol/mg of protein; PR −, ≤10 fmol/mg of protein, as determined by ligand binding assay. Node +, confirmed metastasis in axillary lymph nodes; Node −, absence of metastasis in analyzed axillary lymph nodes. Grade, Nottingham grading system. Subgroup comparison was performed using the Mann-Whitney two-tailed test.

[a] Bold face *P* values correspond to statistically significant differences between tumor subgroups ($P < 0.05$).

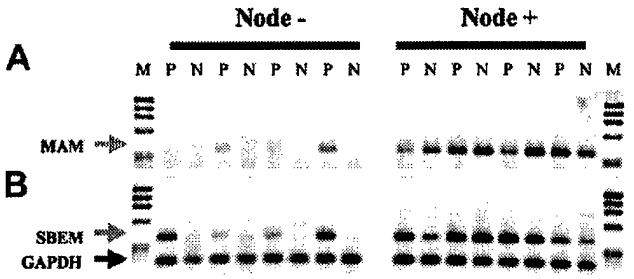

Fig. 2. RT-PCR analysis of *mammaglobin-1, SBEM,* and *GAPDH* mRNA expression in primary breast tumors (*P*) and their corresponding axillary lymph nodes (*N*), histologically shown to contain (*Node +*) or not to contain (*Node −*) metastases. *Mammaglobin-1* (*MAM*) PCR products were run separately (*A, dotted arrow*), whereas *SBEM* and *GAPDH* PCR products were mixed before separation on 2% agarose gels prestained with ethidium bromide (*B*). *Gray arrow,* product corresponding to SBEM; *black arrow,* product corresponding to *GAPDH. M,* molecular weight markers (Φ;x174 RF DNA/Hae III fragments; Life Technologies, Inc., Grand Island, NY). *N,* negative control, no cDNA added during the PCR reaction.

***SBEM* mRNA Expression in Primary Breast Tumors and Their Corresponding Axillary Nodes.** We next investigated the possibility that *SBEM* mRNA could be a tissue marker of axillary lymph node metastasis. Twenty independent cases were selected, including 14 tumors that were axillary lymph node positive and 6 that were node negative. Total RNA was extracted from frozen primary tumor sections and frozen node sections of corresponding axillary lymph nodes. The histological status of all tissues was confirmed in paraffin sections cut from adjacent mirror image paraffin tissue blocks that had been processed in parallel to the frozen blocks. These RNAs were reverse-transcribed and analyzed by RT-PCR using *SBEM*-specific primers. PCR was performed three times, giving the same result. A representative experiment is shown in Fig. 2. A signal corresponding to *SBEM* was detected in all lymph nodes containing metastatic cells by histopathological assessment (14 of 14 cases). In contrast, no signal was detectable in lymph nodes from cases without histologically detectable tumor cells (0 of 6 cases). RT-PCR detection of *SBEM* mRNA in axillary lymph nodes is therefore strongly associated (Fisher's exact test, $P < 0.001$) with the histopathological detection of lymph node metastases. The higher sensitivity afforded by RT-PCR detection therefore indicates that *SBEM,* perhaps together with *mammaglobin-1,* represents an excellent marker for the detection or confirmation of occult breast tumor metastasis, where histopathology may not be definitive.

***SBEM* mRNA Is Expressed in Mammary Epithelial Tumor Cells *in Vivo*.** To further establish whether *SBEM* was expressed by mammary epithelial cells *in vivo*, paraffin breast tumor tissue sections corresponding to a case shown to strongly express *SBEM* mRNA by RT-PCR were studied by *in situ* hybridization. No signal was detectable when using a sense probe (Fig. 3*A*). In contrast, a signal was observed in epithelial tumor cells when using an antisense probe (Fig. 3*B*). *SBEM* mRNA was not detected in stromal or inflammatory cells in any of the sections studied.

Discussion

This article reports the identification, cloning, and preliminary characterization of a cDNA encoding a novel mucin-like protein that displays an unusually narrow pattern of expression. Hybridization analysis revealed that *SBEM* mRNA was only detectable in two normal tissues, breast and salivary gland. Interestingly, the tissue-

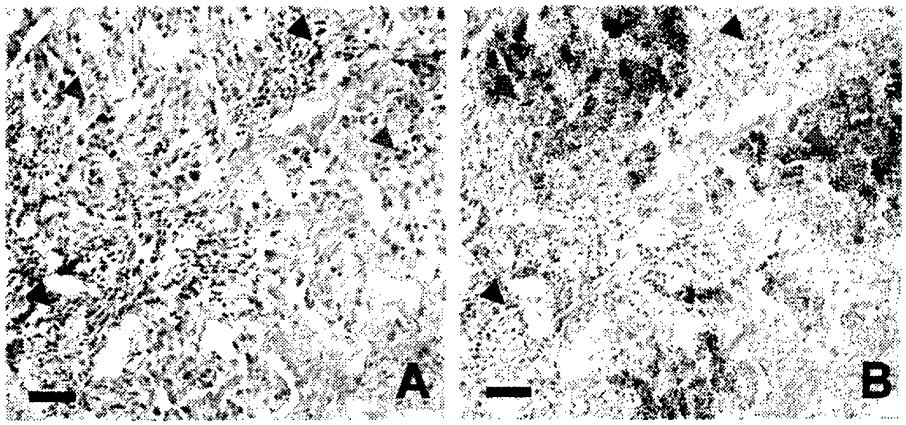

Fig. 3. Expression of *SBEM* mRNA in a primary breast tumor studied by *in situ* hybridization. These plates illustrate consecutive sections from a single breast tumor and show H&E-stained paraffin section treated with a sense probe (*A*) and *SBEM* mRNA expression in epithelial cells detected using an antisense probe (*B*). *Mauve* and *black arrowheads* show tumor epithelial cells and regions of stroma with inflammation, respectively. *Bar*, 30 μm.

specific expression that we observed experimentally directly reflects the distribution of ESTs within the Hs.348419 cluster. Indeed, as mentioned above, only two adult tissues (breast and head/neck tumors) have been shown to express SBEM-related ESTs. The fact that SBEM is also expressed in salivary tissue does not undermine the possible use of SBEM as a marker of breast cancer, because tumors of the salivary gland are less common and can readily be distinguished clinically.

Among the primary breast tumors examined in this study (representing mostly invasive ductal carcinoma), *SBEM* mRNA was observed by RT-PCR analysis in the majority (>90%) of cases. Despite a significant overall correlation between the expression of *SBEM* and *mammaglobin-1* mRNA, a significantly higher SBEM:mammaglobin-1 ratio was observed in primary tumors associated with positive axillary lymph nodes as compared with node-negative tumors. This was mostly attributable to a trend toward higher SBEM expression in node-positive tumors. Although further analysis of a larger number of tumors will be required to confirm these observations, this may suggest differences in the biology of these tumors and also a possible role of SBEM and mammaglobin-1 in the mechanisms involved in tumor metastasis. Our findings indicate, however, that SBEM expression is a common feature of breast cancer and can furthermore serve as a useful marker for breast nodal metastasis, both for detection of micrometastatic cells within lymph nodes as well as in the differential diagnosis of the primary origin of an unknown metastasis. This potential is enhanced by the conserved SBEM expression in high grade and ER/PR-negative tumors that are most likely to metastasize.

The potential diagnostic relevance of SBEM is also increased by its predicted biochemical structure. The *SBEM* cDNA sequence codes for a 90-amino acid polypeptide that contains a distinctive tandem repeat, rich in alanine and threonine residues, that represents a probable target for *O*-glycosylation. Consistent with such posttranslational modification is the presence of a well-defined signal peptide, leading us to predict that SBEM is likely to be processed at the apical surface of luminal epithelial cells and to be secreted into the alveolar or ductal lumen. Further study is needed to ascertain whether higher SBEM expression occurs in association with tumors.

Secreted (or transmembrane) proteins that contain internally repeated, densely glycosylated neutral core motifs such as this are characteristic of mucins, which are typically expressed by the surface epithelium of secretory mucosae and by exocrine glands (14, 15). The role of mucins is primarily one of hydrating and lubricating epithelial linings, although several mucins have been implicated in modulating both cell adhesion and growth factor signaling (18, 19). Furthermore, mucins have a well-established link to cancer, best illustrated by the product of the *MUC1* gene. *MUC1* is overexpressed in a variety of epithelial tumors including breast cancer and gives rise to several well-characterized tumor antigens including CA15.3 and CA27.29 (20, 21). Combined with the loss of cell polarity and changes in glycosylation patterns observed in transformed epithelial cells, overexpression of *MUC1* results in the appearance of mucin-derived tumor antigens in the sera of cancer patients that are not seen in normal controls (14, 22). We hypothesize that a similar situation may hold for SBEM expression in the human mammary epithelium and in human breast tumors. However, *MUC1* displays relatively broad expression among epithelial tissues including the colon, breast, pancreas, ovary, prostate, tracheobronchial tree, stomach, and uterus. For this reason, *MUC1*-derived tumor antigens have relatively poor specificity for individual tumor types, and their clinical utility is limited to monitoring the efficacy of cancer therapy and warning of tumor relapse or malignant spread (21–23).

Parallels between SBEM and known epithelial mucins such as *MUC1,* together with its more narrowly restricted pattern of expression, suggest that this novel gene represents an attractive candidate for a breast biomarker with potential for cancer diagnostics, as well as being a possible future target for the development of a breast tumor vaccine. Moreover, the absence of *SBEM* expression in normal lymph node tissue suggests that this gene could also be used to detect breast micrometastases in axillary lymph nodes.

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Appendix 3 Review, submitted to DNA Cell Biol

Human Small Breast Epithelial Mucin: the promise of a new breast tumor biomarker

Hubé F.[1,2], Mutawe M.[1,2], Leygue E. [2], Myal Y.[1,†]

[1] Department of Pathology, [2] Department of Biochemistry and Molecular Genetic, University of Manitoba, 770 Bannatyne avenue, Winnipeg, MB, R3E 0W3, Canada

Drs Leygue E. and Myal Y. have equally contributed to this work

[†] Corresponding author : Dr Yvonne Myal

Department of Pathology, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, MB, R3E 0W3, Canada. E-mail : myal@cc.umanitoba.ca; Tel : (204) 789-3874; Fax: (204) 789-3931

Running title : hSBEM as a new breast tumor biomarker

Footnotes:

[1] Canadian Cancer Statistics, http://www.cancer.ca/vgn/images/portal/cit_86751114/14/33/195986411niw_stats2004_en.pdf

[2] Breast Cancer Facts and Figures, 2001-2002 http://www.cancer.org/downloads/STT/BrCaFF2001.pdf

[3] NCI Cancer facts, http://cis.nci.nih.gov/fact/

[4] SAGE, Serial Analysis of Gene Expression libraries, http://www.ncbi.nlm.nih.gov/SAGE

Breast cancer remains one of the most frequently diagnosed cancers today. In developed countries, one in eight women is expected to present with breast cancer within her lifetime and an estimated 1,000,000 cases are detected each year worldwide[1]. For women with recurrent disease, the median time of survival is about two years (Ingle et al., 1999). Despite optimal surgery, adjuvant irradiation, hormonal treatment, and chemotherapy, approximately 30% of patients with localized breast cancer finally develop distant metastases. Early detection, which enables intervention at a localized and potentially curable stage, remains a central goal in breast cancer treatment. Indeed, the 5-year survival rate for women with breast cancer has been shown to increase dramatically when the disease is diagnosed at an early stage: from less than 25% in women with disseminated cancer to about 75% in patients with regional disease and over 95% in women with a localized tumor[2]. Unfortunately, only 60% of all breast cancers are diagnosed at a local stage. Any improvement in early detection through identification of tumor biomarkers would have a significant impact on reducing overall breast cancer mortality.

Tumor markers for breast cancer

Tumor biomarkers are defined as molecules/compounds that can be detected in higher-than-normal levels in the body fluids (blood, urine) or tumor tissues of patients affected by the disease[3]. Increased levels of these markers in a given fluid or tissue may result directly from their over-production by the tumor or from the body's response to the presence of this tumor (Cheung et al., 2000; Hayes et al., 2001). Detecting and/or monitoring these changes might assist in the evaluation of cancer risk, diagnosis, or potential response to treatment. Numerous molecules, which hold promise for breast cancer eradication are currently being studied.

Among tumor biomarkers, the estrogen receptor (ER), members of the human epidermal growth factor receptor family (HER-2, HER-2/neu or c-erbB, and epidermal growth factor receptor type 1 or EGFR), and the human prolactin-inducible protein (hPIP/GCDFP-15), are presently being used to diagnose and manage breast cancer patients.

A high level of the ER in the tumor tissue is an overall good prognostic indicator. Indeed, patients with ER-positive tumors, versus those with ER-negative tumors, have not only prolonged disease-free survival after primary treatment, but also have longer and overall superior survival rates (Hilf et al., 1980; Shek et al., 1988; Yoshida et al., 1997). ER can also predict the likelihood of response to hormonal adjuvant therapy such as tamoxifen. About 50 to 60 percent of patients with significant amounts of ER in their tumors respond favorably to hormone therapy (Zhu et al., 1997). An even higher percentage of patients will respond if ER levels are high or if both estrogen and progesterone receptors are positive (Mason et al., 1983).

HER-2 is a type 2 cellular transmembrane tyrosine kinase receptor belonging to the epidermal growth factor receptor family. Its overexpression, not only in breast cancer, but also in other epithelial malignancies such as lung, ovarian or gastrointestinal tumors, is associated with poor prognosis (Pegram et al., 1998; Hynes and Stern, 1994). The gene amplification and/or protein

overexpression of HER-2 was recently found to predict breast cancer outcome in 73 out of 81 studies (90%) that included a total of more than 25,000 patients (Ross et al., 2003). Furthermore, accumulating evidence suggests that HER-2 could also predict the potential response to adjuvant chemotherapy (Paik and Park, 2001). Another interesting observation is that HER-2 extracellular domain is shed into extracellular stroma and can be detected in serum and plasma (Stearns et al., 1998). EGFR, another member of the epidermal growth factor receptor family, is overexpressed in more than 40% of breast cancer (Klijn et al., 1992). Several independent analyses showed that the expression of EGFR in axillary nodes could be an even better indicator than ER to predict the relapse or survival of the patients (Nicholson et al., 1990; Aziz et al., 2002). Still further, the combination of a positive expression of this receptor together with an absence of expression of ER, was shown to have a poorer prognosis (Klijn et al., 1992; Fox et al., 1994).

The hPIP/GCDFP-15 (human prolactin-inducible protein; Shiu and Iwasiow, 1985; Haagensen and Mazoujian, 1986; Myal et al., 1991) was originally investigated as a clinical marker to diagnose metastatic tumors of unknown origin. hPIP/GCDFP-15 expression positively correlates with estrogen receptor status and is frequently observed in both primary human breast tumors and nodal metastases (Haagensen and Mazoujian, 1986; Murphy et al., 1987; Clark et al., 1999).

Other biological markers are still currently extensively studied. The mucin 1 (MUC1; Taylor-Papadimitriou et al., 1999; von Mensdorff-Pouilly et al., 2000) and the mammaglobin (Watson and Flemming, 1996) are holding the promise to become established prognostic and/or predictive factors.

With a size of over 1200 amino acids, the MUC1 protein is very large, polymorphic and is heavily glycosylated (Marth and Varki, 1999; Baruch et al., 1997). It was found to be over-expressed in many different epithelial tumors including breast and lung cancers. MUC1 related tumor antigens including CA15.3 and CA27.29, which correspond to different glycosylated forms of MUC1 protein (Zimmerman et al, 2000; Cheung et al., 2000) can be detected in the sera of cancer patients, but not in

normal controls (Marth and Varki, 1999). It has been hypothesized that the non-glycosylated form of MUC1, only detected in breast cancer, may play a role in the initial attachment of carcinoma cells to tissues at distant sites, facilitating establishment of metastatic foci (Ciborowski and Finn, 2002).

Mammaglobin, a previously described breast specific gene (Watson and Flemming 1996; Leygue et al., 1999; Zach et al., 1999) is over-expressed in many breast tumor tissue compared to corresponding normal breast tissue and its expression correlates with estrogen receptor status (Miksicek et al., 2002; Guan et al., 2003). In addition, mammaglobin has successfully been used to detect breast cancer cells in axillary lymph nodes and blood (Leygue et al., 1999; Suchy et al., 2000). Such data suggest a potential for prognosis and for monitoring the evolution or the recurrence of the disease.

Unfortunately, to date, all designated 'breast tumor markers' are not primarily breast specific and only allows for the characterization of small subsets of patients. There is an urgent need to identify novel genes whose expression is restricted to the mammary epithelium to improve early detection and subsequently reduce death due to breast cancer. Indeed, such genes have the greatest potential (alone or combined with existing markers) not only to report on proliferative changes in the breast, which may herald increased risk of breast cancer development, but also to enhance the detection of micrometastatic disease, and to be used for targeting specifically breast cancer cells.

Identification of the human Small Breast Epithelial Mucin (hSBEM)

In recent years, an increase in new technologies and the progress of the genome sequencing project has led to the development of methods allowing large scale studies of DNA expression and the establishment of tissue-specific gene expression profiles. Among these new tools, cDNA/protein microarrays (Nacht et al., 1999; Li et al., 2002), subtractive hybridization (Quan and Lu, 2003; Ermolaeva and Sverdlov, 1996) and SAGE (Serial Analysis of Gene Expression, Velculescu et al., 1995; Nacht et al., 1999) are the most commonly used.

Such approaches led our laboratory (Miksicek et al., 2002) and 2 others independent laboratories (Houghton et al., 2001; Colpitts et al., 2002) to the concomitant identification of a new breast specific gene, reported by the different authors as B511s, hSBEM and BS106. B511S was identified using cDNA library subtraction/DNA microarray whereas hSBEM and BS106 were identified by SAGE analysis. For the purpose of this review, the name hSBEM will be used, as it defines the first sequence entered in Genbank (#AF414087).

hSBEM: Gene, mRNA and protein structure

The hSBEM gene, located on chromosome 12q13, spans a 3.9 kb region consisting of 4 exons and 3 introns (Fig. 1). The transcript of 600-700 bp (Miksicek et al., 2002; Colpitts et al., 2002) (GenBank #AF414087) encodes for a protein of 90 amino acids. The first 19 amino acids has all the characteristics of a signal peptide, indicating that hSBEM protein is likely a secreted protein (Colpitts et al., 2002; Miksicek et al., 2002; Clark et al., 2003). This assumption has now been confirmed through the detection of the hSBEM protein in cell culture medium (Colpitts et al., 2002) as well as through the recent identification of this molecule by the Secreted Protein Discovery Initiative (Clark et al., 2003).

hSBEM protein is rich in Ala (21%), Thr (18%) and Pro (11%) residues, many of which are organized as an octapeptide core motif (TTAAXTTA) repeated three times. Such motifs are also found in mucins and led us originally to name this new breast specific gene, human small breast epithelial mucin or hSBEM (Miksicek et al., 2002). In the absence of any post-translational modifications, the theoretical size of hSBEM protein is 9 kDa. Interestingly, a much higher molecular weight, ranging between 17 and 70 kDa, has been experimentally observed (Colpitts et al., unpublished observations). These higher molecular weights may result from extensive O-glycosylations through the Thr-rich sequence. This hypothesis is further corroborated by the binding of hSBEM to lectins, molecules which recognize galactose or N-acetyl-galactose present on O-glycosylated chains (Colpitts et al., 2002). Moreover, beside glycosylations, additional post-translational modifications such as dimerization involving cysteine residue present in the C-terminal could also participate in the observed increased molecular weights.

hSBEM: gene expression

Database analysis

Database analysis of SAGE (Serial Analysis of Gene Expression) libraries[4] shows that amongst more than 2000 Tags corresponding to hSBEM mRNA (Unigene #348419, Tag: CTTCCTGTGA), only 17 (0.6%) are found in tissues other than breast (Fig. 2). No tags corresponding to hSBEM are detected in cDNA libraries from bone marrow, brain, cerebellum, ovary, kidney, liver, pancreas, skin, stem cells, stomach, testis, or thyroid. In contrast, about 30%, 60% and 25% of tags corresponding to known markers HER2, MUC1 and mammaglobin transcripts, respectively, are found in non-breast tissues. Overall, two years after its identification as a putative breast specific gene, database analysis still suggests that hSBEM expression remains mainly confined to this tissue.

Normal tissues

Several groups have now reported the experimental detection of hSBEM mRNA in normal tissues (Houghton et al., 2001; Miksicek et al., 2002; Colpitts et al., 2002). Results of these studies are summarized Table 1. Northern dot blot analyses revealed hSBEM mRNA expression only in breast and salivary glands, whereas all other normal tissues such as brain, ovary, uterus, prostate, or lung were negative (Miksicek et al., 2002; Colpitts et al., 2002). Using more sensitive techniques such as microarray hybridizations and RT-PCR, it was found that colon, kidney, heart, skin and lung were the only other normal tissues expressing low but detectable levels of hSBEM mRNA (Houghton et al., 2001; Koga et al., 2004). The absence of any detectable signal in normal axillary lymph node, even when using RT-PCR, is of particular interest, as such characteristic is indeed advantageous for

the detection of micrometastases. The detection of the hSBEM protein expression in normal breast has also now been successfully achieved using immunohistochemistry (Colpitts et al, 2002).

Altogether experimental data has confirmed the database analysis: a strong expression of hSBEM in normal breast, and low or undetectable expression in other normal tissues.

Tumor tissues

As shown Table 1, hSBEM mRNA expression has been detected by RT-PCR in most breast cancer cells lines and in more than 90% of breast tumors studied (Miksicek et al., 2002; Colpitts et al., 2002). To date, the prostate is the only non-breast tumor tissue shown experimentally to express hSBEM detectable levels mRNA (Colpitts et al., 2002). This is not altogether surprising as prostate and breast often conserve similar gene expression. For example, prostate specific antigen, which is 'specific' in prostate tissues, is also expressed in breast tissues (Mannello and Gazzanelli, 2001). Immunohistochemical study performed by Colpitts et al. confirmed the presence of hSBEM protein in most breast tumors studied but not in any other tumor tissues such as colon, lung or ovary. These authors also observed an increase expression of this protein in poorly differentiated breast tumors.

hSBEM: promises as a breast tumor biomarker

The extremely narrow pattern of expression of hSBEM together with its conservation during breast tumorigenesis and tumor progression provides a unique opportunity for the clinician to detect and target breast cancer cells. The study of hSBEM could lead to several different applications (Fig. 3).

Detection of breast metastases

We have successfully detected breast metastases in axillary lymph nodes by RT-PCR amplification of hSBEM cDNA (Miksicek et al., 2002). Consequently, we have suggested that hSBEM could be used to detect micrometastases potentially missed during routine histological analysis. Recently, Weigelt et al. (2004) confirmed that the concomitant use of multiple markers such as hSBEM and CK19 could indeed allow the detection of such micrometastases in sentinel lymph nodes. Even though such use of hSBEM is potentially clinically relevant, some limitations to this approach should be considered. The RT-PCR detection of breast micrometastases in any given tissue will not be possible if the tissue express low levels of hSBEM. As a result, this strategy could not be used to detect breast metastases in tissues like lungs (Houghton et al., 2001). However, in these tissues, the analysis of hSBEM protein expression by immunohistochemistry may be an alternative strategy for the detection of breast micrometastases.

Monitoring the appearance or the evolution of the disease

Monitoring circulating levels of markers detectable in body fluids would be of tremendous value and could be applied to several situations. We could envision for example the determination of risk for developing cancer, screening programs for early detection or distinguishing benign from malignant states. However, to date, the perfect circulating tumor marker for breast cancer cells has

not yet been identified. Some cancer protein markers like the HER-2 extracellular domain or intercellular adhesion molecule 1 (ICAM-1) are shed in serum and plasma and measure of their levels could be used to monitor the progression of the disease. However, the discrimination between normal and overexpressed levels is often difficult to achieve. For example, normal serum levels of soluble ICAM-1 is 346 ± 68 ng/mL while tumor levels 463 ± 92 ng/mL. In addition, most tumors markers are also widely expressed in normal tissues (Stearns et al., 1998) such as prostate, colon, lung and pancreas. Thus, a secreted protein like hSBEM, specifically expressed in breast, could be potentially more useful. We hypothesize that the secretion of hSBEM protein by cancer cells will lead to its increased concentration in the blood of breast cancer patients. Monitoring blood hSBEM protein levels, using ELISA, for example, may therefore allow the clinician to detect the presence of breast cancer cells (with all the advantages of an early detection) or to follow/monitor the response to a given therapy. A similar strategy has been used successfully for HER-2 by Meenakshi et al. (2002) who developed an ELISA for quantitation of serum HER-2. However, these authors showed that only 20% of breast cancer patients had elevated levels of circulating HER-2.

Targeting breast cancer cells

Ideally, the clinician would benefit from therapy that specifically targets only breast cancer cells. The use of antibodies as vectors that would recognize breast cancer cells and deliver therapeutic genes or cytotoxic drugs has already been explored (Frankel, 1993; Cheng, 1996; Lee et al., 2003). For example, drugs directly immunoconjugated to HER-2 antibody were used to target cell surface receptors on breast cancer (Punj et al., 2004). Some other laboratories have used HER-2 antibody or a ligand protein, transferrin, conjugated to cationic liposomes to specifically target tumoral cells and increase the low transfection efficiency with liposomes alone (Cheng, 1996; Lynch et al., 1997). Today, no reliable strategy has yet been successfully developed, likely because of the lack of specific

delivery. To this end, anti-hSBEM antibodies immunoconjugated with toxic reagents could be more clinically useful. The antibodies would surround the breast cancer cells and subsequently the drug could be directly delivered on site.

Gene-specific therapy

An alternative approach to specifically delivering a gene to the tumor site is the use of gene-specific therapy. This strategy has been used with Cox-2 and HER-2 promoters (Hurst, 2001; Godbey and Atala, 2003). On one hand, the Cox-2 gene is overexpressed in many cancers including colon, pancreas, prostate, bladder and 50% of breast cancers (Spizzo et al., 2003; Godbey et Atala, 2003). On the other hand, HER-2 is overexpressed in about 30% of breast and ovarian cancers. Transcriptional regulation of HER-2 expression was extensively studied (Hudson et al., 1990; Zhao and Hung, 1992; Chang et al., 1997) and the HER-2 promoter regions have been used in 'prodrug-activation therapy' to selectively express a suicide gene in tumor cells. A phase I clinical trial in 12 patients was also performed, demonstrating the selectivity of such approach (Pandha et al., 1999). Sequence analysis of hSBEM promoter should allow us to identify regions responsible for the breast specific expression of this gene. Constructs containing these regulatory regions driving the expression of a gene inducing cell death could be generated and artificially introduced in cancer tissues through viral infection. These constructs, inactive in non-breast cells, could selectively express the cytotoxic gene and subsequently be used to destroy breast cancer cells (Dachs et al., 1997; Wilcox et al., 1999).

Overall, hSBEM holds much promise to become a clinically relevant molecule for breast cancer detection and follow up. Indeed, its tissue-specific expression makes it a better candidate than most other markers currently used. Further studies are being currently undertaken to explore this potential.

Acknowledgments

E.L. is the recipient of a USAMRMC Career Development Award grant number DAMD17-01-1-0308, and Y.M. is a Manitoba Medical Service Foundation Career Awardee. This work is supported by, and the Canadian Institute of Health Research (CIHR # MOP 62971) and Friends You Can Count On.

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Table 1.

	Dot blot	RT-PCR	IHC
Normal tissues			
Breast	+	+++	+++
Salivary gland	+++	+++	
Skin	-	++	+/-
Lung	-	++	-
Lymph node	-	-	
Colon	-	-	-
Bladder	-	-	-
Uterus	-	-	-
Stomach	-	-	-
Prostate	-	-	-
Ovary	-	-	-
Pancreas	-	-	-
Kidney	-	-	-
Liver	-	-	-
Fetal tissues	-		
Cancer cells and tissues			
Breast	+++	+++	+++
Prostate	+	-	-
Colon			-
Bladder			-
Uterus		-	-
Gastric			-
Ovary			-
Pancreas			-
Liver		-	-
Lung			-

Figure legends

Table 1. Expression of hSBEM in normal and tumoral tissues.

Dot blot, Northern dot blot; RT-PCR, Reverse transcription-polymerase chain reaction; IHC, Immunohistochemistry. +++, + or -designate a strong expression, a weak expression or none expression detected, respectively. The blanks indicate that the tissue were not tested by the technique.

Fig. 1. Gene, mRNA and protein structure of hSBEM.

On the top, a schematic representation of the hSBEM genomic structure. The number referred to the chromosomal numbering. Hs. 348419 is the name of the cluster regrouping all the ESTs sequences corresponding to hSBEM. On the middle panel, the hSBEM mRNA is composed of 4 exons, the first of which containing the ATG. Because the transcription start site is not known yet, the numbering is given accordingly to the published sequence (Genbank #AF414087). On the bottom, the protein sequence (Genbank #AAL02119) of hSBEM is composed of 90 amino acids. The putative secreted signal in N-terminus is underligned, the 3 repeats (TTAAXTTA) in the neutral core are boxed, and the putative O-glycosylation sites are indicated by stars.

Fig. 2. SAGE analysis of hSBEM, Mammaglobin, MUC1 and HER-2.

The graph is a representation of the number of Tags identified in breast libraries (black) compared to others libraries (gray). 2161, 397, 1180 and 790 Tags corresponding to hSBEM, Mammaglobin, MUC1 and HER-2 were identified in 40, 87, 108, 133 libraries, respectively. In these, 2144, 300, 525

and 501 Tags corresponding to hSBEM, Mammaglobin, MUC1 and HER-2 were identified in 31, 28, 31 and 31 breast libraires, respectively.

Fig. 3. Possible use of hSBEM in therapeutic.

Several different applications could, in the future, arise from the study of hSBEM. Amon them, the detection of breast metastases in lymph nodes, the monitoring of the disease, targeting of breast cancer cells with specific antibodies or the delivery of therapeutic genes under the promoter-specific expression.

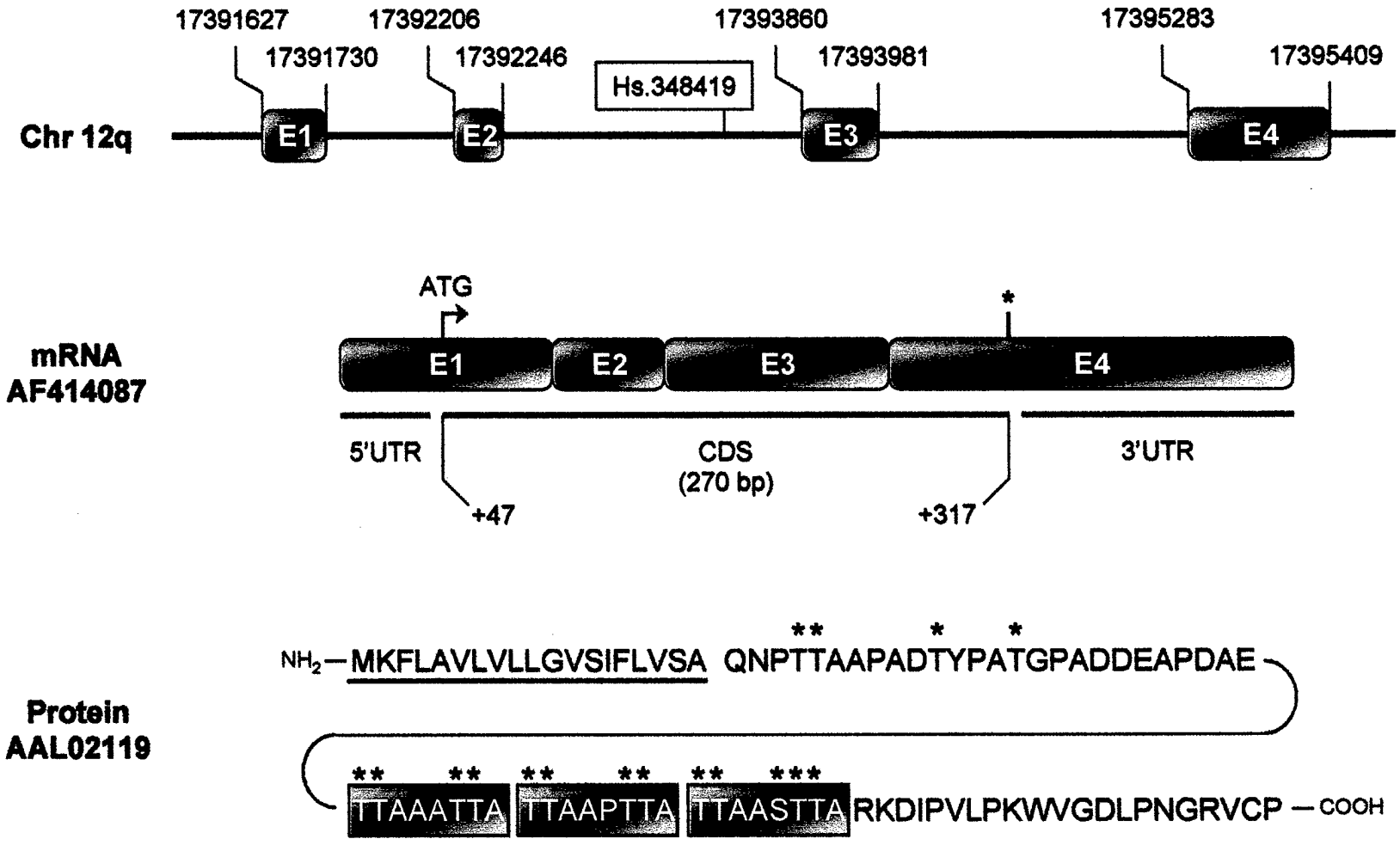
17391627
17391730
17392206
17392246
17393860
Hs.348419
17393981
17395283
17395409
Chr 12q
E1
E2
E3
E4
ATG
mRNA
AF414087
E1
E2
E3
E4
5'UTR
CDS
(270 bp)
3'UTR
+47
+317
Protein
AAL02119
NH2—MKFLAVLVLLGVSIFLVSA QNPTTAAPADTYPATGPADDEAPDAE
TTAAATTA
TTAAPTTA
TTAASTTA
RKDIPVLPKWVGDLPNGRVCP —COOH

Figure 1

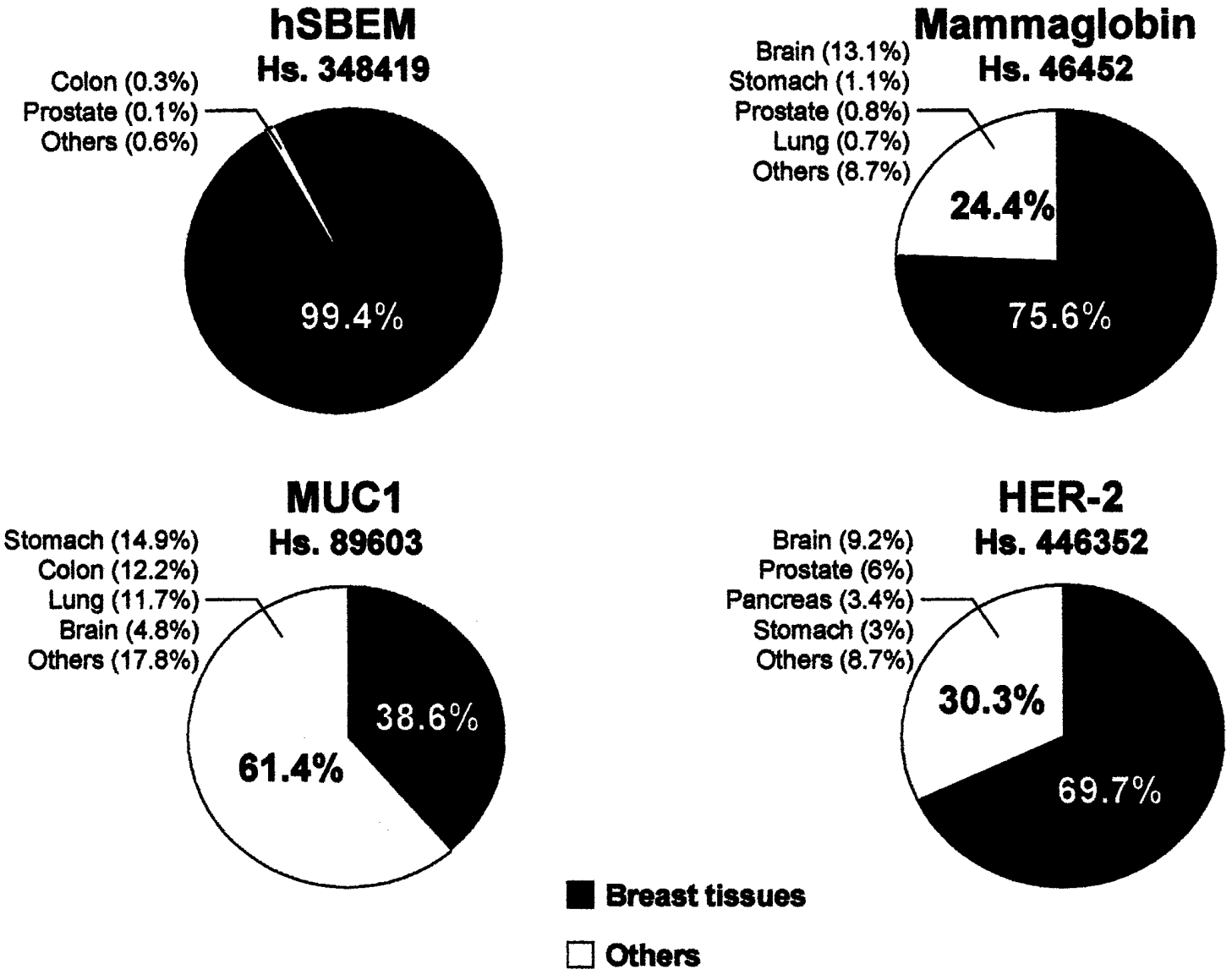
hSBEM
Hs. 348419
Colon (0.3%)
Prostate (0.1%)
Others (0.6%)
99.4%
Mammaglobin
Hs. 46452
Brain (13.1%)
Stomach (1.1%)
Prostate (0.8%)
Lung (0.7%)
Others (8.7%)
24.4%
75.6%
MUC1
Hs. 89603
Stomach (14.9%)
Colon (12.2%)
Lung (11.7%)
Brain (4.8%)
Others (17.8%)
38.6%
61.4%
HER-2
Hs. 446352
Brain (9.2%)
Prostate (6%)
Pancreas (3.4%)
Stomach (3%)
Others (8.7%)
30.3%
69.7%
Breast tissues
Others

Figure 2

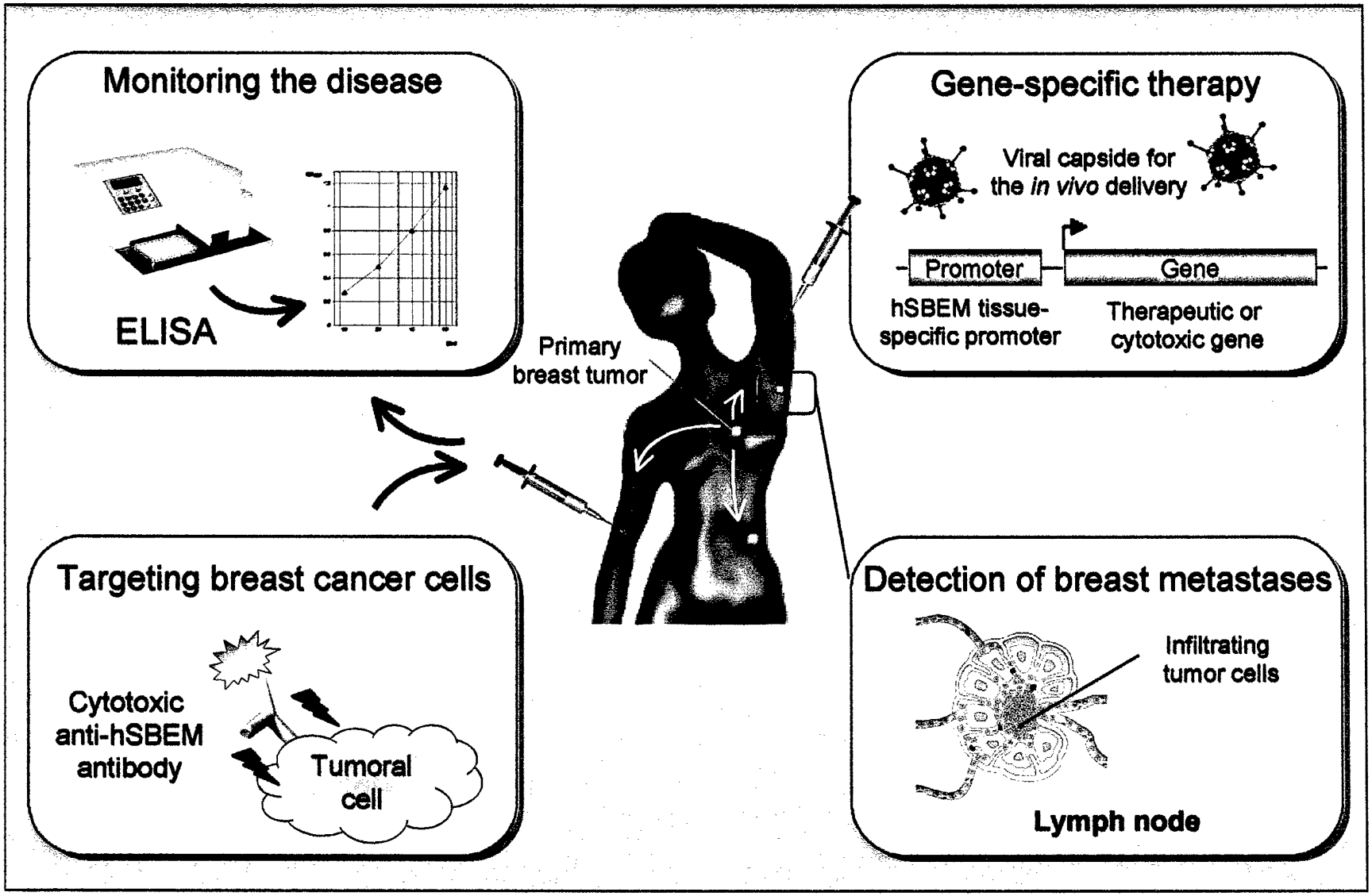
Monitoring the disease
ELISA
Gene-specific therapy
Viral capside for the *in vivo* delivery
Promoter
Gene
hSBEM tissue-specific promoter
Therapeutic or cytotoxic gene
Primary breast tumor
Targeting breast cancer cells
Cytotoxic anti-hSBEM antibody
Tumoral cell
Detection of breast metastases
Infiltrating tumor cells
Lymph node

Figure 3

Appendix 4 Abstract #3282. AACR meeting 2004

Abstract Number: 3282

SBEM protein expression correlates with estrogen receptor and tumor grade in breast cancer

Mark M. Mutawe, Etienne Leygue, Yvonne Myal. University of Manitoba, Winnipeg, MB, Canada.

We recently identified small breast epithelial mucin (SBEM) as a novel 90 amino-acid protein, the expression of which appears to be highly breast-specific. Structurally, this protein contains tandem repeats of the octapeptide neutral core sequence TTAAXTTA, that is characteristic of the large family of glycoproteins known as mucins. Since the epithelial surface mucin, MUC1, a member of this family, is abnormally glycosylated and its expression is up-regulated in breast cancer, we were interested in examining protein expression of SBEM in breast cancer cell lines, normal breast tissue and breast tumors. Western blot analysis of eight different breast cancer cell lines indicated variable SBEM protein expression. The MDA-231 and BT-20 cell lines exhibited high expression of SBEM protein, while the T5 and MCF-7 cell lines expressed SBEM at lower levels. In normal mammary tissue, SBEM was present, but there was a low level of expression. 88 breast tumors were analyzed by Western blot, and were classified as being SBEM-positive (n = 64) or SBEM-negative (n = 24). When compared to ER levels, SBEM positive tumors demonstrated lower ER levels (22.5fmol/mg) than SBEM negative tumors (63.0fmol/mg). There was also a positive correlation between SBEM level and tumor grade (P = 0.02). No correlation between SBEM and PR levels was observed (P = 0.71). All other normal tissues studied were negative for SBEM protein. These data suggest that a higher expression of the SBEM protein could help monitor breast cancer progression.

Presenter: Mark M. Mutawe

Affiliation: University of Manitoba, Winnipeg, MB, Canada; E-mail: markmutawe@hotmail.com

 Citation information: Proceedings of the AACR, Volume 45, March 2004.

Appendix 5 Abstract, submitted San Antonio Breast Cancer Meeting, 2004

Functional analysis of the promoter for the breast-specific gene SBEM

Hube F, Hamedani MK, Mutawe M, Chooniedass-Kothari S, Myal Y, Leygue E.
Department of Biochemistry and Medical Genetics and Department of Pathology, University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, Canada, R3E 0W3

We recently identified a highly breast-specific gene that we have termed human small breast epithelial mucin (SBEM). To elucidate the molecular mechanisms underlying the tissue-specific transcriptional regulation of SBEM, we are currently studying the SBEM gene promoter. A 1-kb human SBEM gene 5'-flanking region was isolated, cloned and sequenced. Promoter region contains two typical mammalian overlapping TATA boxes, suggesting the presence of two possible transcription initiation sites. Subsequently, these sites were experimentally localized by 5'-RACE PCR in -69 and -67 upstream from the translation initiation site. Using TransFac database, several transcription factor binding sites (GATA, Oct, NFY, RFX, ...) were identified. Interestingly, some of these were clustered in a "hormone-response region" (half binding-sites for estrogen receptor, Sox9, RORalpha). To explore the importance of these transcription factor and to identify regions responsible for the breast-specificity of SBEM expression, a series of luciferase reporters driven by different size fragments from the SBEM promoter were constructed. These constructions were then used to transfect transiently both mammary and non-breast cells lines. In addition of the minimal promoter located in the –132/-106 region, we identified a 87 bp sequence (-357/-270) which is responsible for a strong expression in mammary cancer cells only. Identification of breast-specific sequences could facilitate the targeting of breast cancer cells for therapeutic genes delivery.

Appendix 6 Cancer Res. 2003. 63: 1954–1961

[CANCER RESEARCH 63, 1954–1961, April 15, 2003]

Psoriasin Interacts with Jab1 and Influences Breast Cancer Progression[1]

Ethan D. Emberley, Yulian Niu, Etienne Leygue, Ladislav Tomes, R. Daniel Gietz, Leigh C. Murphy, and Peter H. Watson[2]

Departments of Pathology [E. E., Y. N., L. T., P. H. W.] and Biochemistry and Medical Genetics [E. L., R. D. G., L. C. M.], University of Manitoba, Winnipeg, Manitoba R3E 0W3, Canada

ABSTRACT

Psoriasin (S100A7) is expressed at low levels in normal breast epithelial cells but is highly expressed in preinvasive ductal carcinoma *in situ*. Persistent psoriasin expression occurs in some invasive carcinomas and is associated with poor prognostic factors. Whereas there is evidence that secreted psoriasin can act as a chemotactic factor for CD-4-positive lymphocytes in psoriatic skin lesions, an intracellular biological function is unknown. We have found that psoriasin physically interacts with Jab1 (c-jun activation-domain binding protein 1) in the yeast two-hybrid assay and confirmed this by coimmunoprecipitation assay in breast cancer cells. Psoriasin-transfected breast cancer cells showed increased nuclear Jab1 and demonstrated several features consistent with an alteration in Jab1 activity including an increase in activator protein-1 (AP-1) activity, increased expression of AP-1 and *HIF-1*-dependent genes, and reduced expression of the cell-cycle inhibitor $p27^{Kip1}$. Psoriasin overexpression was also associated with alteration of cellular functions that are associated with increased malignancy, including increased growth, decreased adhesion, and increased invasiveness *in vitro*, as well as increased tumorigenicity *in vivo* in nude mice. We conclude that intracellular psoriasin influences breast cancer progression and that this may occur through stimulation of Jab1 activity.

INTRODUCTION

We have identified psoriasin (S100A7) previously as a differentially expressed gene between DCIS[3] and invasive carcinoma (1). The expression of psoriasin is low in normal breast and benign pathologies (1), but psoriasin is among the most highly expressed genes in high grade DCIS (2, 3). Whereas expression is often reduced in invasive carcinoma, persistent high expression is associated with markers of poor prognosis (4). This profile of gene expression raises the possibility that psoriasin may be functionally involved in invasion and early tumor progression (5). Psoriasin is a small calcium-binding protein belonging to the S100 gene family (6, 7), among which several other members have been associated with breast tumor progression (8, 9). Most interest has been focused on *S100A4* (10). which was also initially identified as a differentially expressed gene between nonmetastatic and metastatic rodent mammary tumor cell lines (11). In later studies by several groups, *S100A4* has been shown to directly influence the invasive and metastatic phenotype in breast cancer cell lines (12–14) and tumors (15), and expression is also associated with poor prognostic factors and patient survival in human breast tumors (16).

Psoriasin was originally described as highly expressed within psoriatic skin lesions (17) and found to be a secreted protein (18), but has since been observed to be present in the cytoplasm and nucleus of both abnormally differentiated keratinocytes (19) and breast carcinoma cells (2, 4). Whereas there is evidence that secreted psoriasin can act as a chemotactic factor for CD-4-positive lymphocytes in psoriatic skin lesions (18), a function for intracellular psoriasin also appears likely but has yet to be established.

We sought to identify proteins that might interact with psoriasin in breast epithelia by using the yeast two-hybrid system (20). Jab1 (21) was found to specifically interact with psoriasin in the yeast system, and this interaction was confirmed by biochemical assay in breast cancer cells. Jab1 is a component of a multimeric protein complex (22, 23), the CSN/COP9 signalosome, which is involved in signal transduction and protein degradation via the Ub-26S proteasome (24, 25). The effect of overexpression of psoriasin on Jab1 distribution and function in a breast cancer cell line was studied. Psoriasin overexpression resulted in redistribution of Jab1 to the nucleus and multiple functional changes that can be attributed to activation of Jab1, as well as enhanced tumorigenesis and metastasis in an *in vivo* assay. These data support our hypothesis that psoriasin enhances early tumor progression and the process of invasion in breast cancer cells in part by interacting with Jab1 and positively enhancing its activity.

MATERIALS AND METHODS

Yeast Two-Hybrid System. For yeast two-hybrid studies, the coding region of human psoriasin protein was fused in-frame with the GAL4 DNA-binding domain of the pGBT9 vector (Clontech). The resulting bait plasmid (pGBT9-psor) was used to screen a normal human mammary epithelium cDNA library (Clontech) by the yeast two-hybrid method as we have described previously (26). Clones were isolated that could grow on Trp^- Leu^- His^- medium, did not autoactivate the β-galactosidase reporter gene, and demonstrated specificity for their interaction with psoriasin. This was done by testing the interaction of psoriasin with specific "prey" constructs not identified in the screen. Jab1 was analyzed in a similar fashion. The NH_2-terminal "bait" psoriasin plasmid used to define the region of psoriasin involved in Jab1 binding encoded amino acids 1–52 (pGBT9-N-term-psor), and the COOH-terminal bait psoriasin plasmid encoded acids 43–101 (pGBT9-C-term-psor).

Cell Culture, Transfections, and Antibodies. The human breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 were cultured in DMEM supplemented with 10% FBS under standard conditions (4). The former cell line is negative for psoriasin, whereas the latter expresses psoriasin mRNA and protein (confirmed by RT-PCR and Western blot; data not shown). The full psoriasin protein coding sequence was cloned into pcDNA3.1 (Invitrogen) and transfected into MDA-MB-231 cells using Superfect (Qiagen) followed by G418 selection. Resistant colonies were isolated and expanded. Psoriasin protein expression was determined by Western blot using a rabbit antipsoriasin antibody generated by our laboratory and directed against the epitope KQSH-GAAPCSGGSQ corresponding to amino acids 88–101. The specificity of the antibody was established by comparison with a similar antibody generated previously against the same epitope (4), and by immunohistochemistry and Western blot, using transfected breast cancer cell lines and tumors as described previously (4). Three MDA-MB-231 clones were found to express psoriasin

Received 8/13/02; accepted 2/19/03.

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[1] Supported in part by the Canadian Breast Cancer Research Initiative Grant 11147, the United States Army Medical Research and Materials Command Grant DMAD 17-97-1-7320 and the Canadian Institutes for Health Research. P. H. W. is the recipient of a United States Army Medical Research and Materiel Command Academic Award Grant DMAD 17-99-1-9272 and a Canadian Institutes for Health Research Scientist Award. E. L. is the recipient of a United States Army Medical Research and Materiel Command Career Development Award Grant DMAD 17-01-0308. E. D. E. is the recipient of a United States Army Medical Research and Materiel Command Predoctoral Traineeship Grant DAMD 17-00-1-0320.

[2] To whom requests for reprints should be addressed, at Department of Pathology, University of Manitoba, D212-770 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W3, Canada. E-mail: pwatson@cc.umanitoba.ca.

[3] The abbreviations used are: DCIS, ductal carcinoma *in situ*; RT-PCR, reverse transcription-PCR; AP-1, activator protein; VEGF, vascular endothelial growth factor; MMP3, matrix metalloproteinase 13/collagenase3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Jab1, c-jun activator binding protein-1; CAIX, carbonic anhydrase IX.

(designated as clone 231-LP1 exhibiting low psoriasin expression, and clones 231-HP1 and 231-HP2 both exhibiting similar high levels of psoriasin expression). Wild-type MDA-MB-231 and clone 231-neo (generated by transfection with the empty vector) do not express psoriasin. Jab1 and $p27^{Kip1}$ antibodies were obtained from Santa Cruz Biotechnology, Inc. Hypoxic stimulation of cells was performed in a Forma Scientific Model 1025 Anaerobic System containing an atmosphere of 0.7% O_2, 5% CO_2, and 5% H_2 at 37°C for 24 h as we have described previously (27).

Immunoprecipitation and Western Blot. Human breast cancer cell lines expressing psoriasin (231-HP2 and MDA-MB-468) were lysed on ice in 25 mM HEPES (pH 7.7), 0.4 M NaCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 1% Triton X-100, 0.5 mM DTT, and protease inhibitor mixture (Roche). Complexes were immunoprecipitated by Jab1 antibody/protein G-Sepharose (Pierce) at 4°C for 2 h. Binding and washes were performed in the same buffer, except the NaCl concentration was diluted 4-fold (28). Coimmunoprecipitated psoriasin protein was detected by immunoblotting using the psoriasin-specific antibody. Total protein lysates were extracted from the cell-line pellets in SDS-Isolation Buffer [50 mM Tris (pH 6.8), 20 mM EDTA, 5% SDS, 5 mM β-glycerophosphate, and a mixture of protease inhibitors (Roche)]. Protein concentrations were determined using the Micro-BCA protein assay kit (Pierce). Protein lysates were run on a 16.5% SDS-PAGE mini gel using Tricine SDS-PAGE to separate the proteins, and then transferred to 0.2 μm nitrocellulose (Bio-Rad). After blocking in 10% skimmed milk powder in Tris-buffered saline-0.05% Tween, blots were incubated with primary antibodies (~15 μg/ml in Tris-buffered saline-0.05% Tween) followed by incubation with appropriate secondary antibodies and visualization by incubation with Supersignal (Pierce) as per the manufacturer's instructions and exposure on X-ray films.

Reporter Gene Assay and Transcription of AP-1-dependent Genes. MDA-MB-231 parental cells and clones stably transfected with psoriasin were transfected with an AP-1-driven luciferase reporter gene (Stratagene) and a β-galactosidase expression vector, in triplicate experiments using Effectene (Qiagen). Luciferase was measured in cell lysates (Promega) 18 h after transfection and standardized to β-galactosidase activity (Promega). Total RNA from the MDA-MB-231 clones was isolated using TRIzol (Sigma) and reverse transcribed in duplicate from triplicate samples as described (4). Specific primers for VEGF, MMP13, and GAPDH were used for PCR as follows: VEGF-UPPER (sense) CGC AGA CGT GT AAA TGT TCC and VEGF-LOWER (antisense) AAG AAA AAT AAA ATG GCG AAT CC; MMP13-UPPER (sense) ATG CGG GGT TCC TGA T and MMP13-LOWER (antisense) CGC AGC AAC AAG AAA CAA; and GAPDH-UPPER (sense) ACC CAC TCC TCC ACC TTT G and GAPDH-LOWER (antisense) CTC TTG TGC TCT TGC TTG TTG G. Reactions were stopped during the log-linear stage of PCR amplification and samples electrophoresed through an agarose gel that was poststained with ethidium bromide for band visualization. Images were captured using an LCD camera and MCID software (Imaging Research, St. Catharine's, Ontario, Canada).

Immunohistochemistry. Cultured cells were grown on microscope slides for 24 h, and then fixed and processed as described previously (4). Immunohistochemical staining for psoriasin was performed essentially as described previously, using an automated tissue immunostainer (Ventana Medical Systems, Phoenix, AZ), and 3,3′-diaminobenzidine immunohistochemistry kit and bulk reagents supplied by manufacturer. Briefly, the staining protocol was set to "Extended Cell Conditioning" procedure, followed by 12 h incubation with primary antibody (concentration 1:3000) and 32-min incubation with secondary antibody. Positive staining was assessed by light microscopy.

Adhesion, Growth, and Invasion Assays. MDA-MB-231 clones were trypsinized from flasks that were 60–70% confluent. Cells (10,000) were plated in triplicate on three different days in 96-well plates having fibronectin, collagen I, or uncoated plastic surfaces (Becton Dickinson). After 1 h at 37°C, nonadherent cells were gently washed away with PBS. Adherent cells were stained with crystal violet, and their relative abundance determined by spectrophotometric absorbance. For growth assay, 1000 cells/well were plated in plastic 96-well plates in triplicate on three different days and allowed to grow for 18, 24, 48, and 72 h. Cells were stained with crystal violet and their relative abundance determined by spectrophotometric absorbance. Invasion assays were preformed in triplicate on a Matrigel-coated modified Boyden-invasion chamber (24-well plate inserts with 8-μm pores; Becton Dickinson). FBS DMEM (10%) was used as a chemoattractant in the lower chamber. Cells (350,000) were added to the upper chamber, and allowed 12 h to degrade the Matrigel and invade through the porous membrane. Cells that invaded and were adhering to the bottom of the membrane were stained with crystal violet. Invaded cells were visualized by light microscopy and numerated by counting the number of cells per high power field in five random fields.

***In Vivo* Mouse Studies.** Breast cancer cells (four experimental groups comprising MDA-MB-231 parental cells, 231-neo control, 231-LP1, and 231-HP1) were grown in culture and then suspended in 0.2 ml of PBS at a concentration of 5×10^5 cells before injection into mammary fat pads of female nude mice according to a protocol approved by the University of Manitoba Animal Care Committee. Each experimental group included 5 animals, and two injections were sited bilaterally in each animal to achieve a total of 10 possible tumor sites per group. Tumor diameters were measured by calipers at weekly intervals, and the tumor volume was calculated from the formula: volume = $4/3\ \pi\ (0.5 \times \text{smaller diameter}^2 \times 0.5 \times \text{larger diameter})$. The experiment was continued for up to 8 weeks at which time all of the animals were euthanized, and all of the injection sites, tumors, and multiple organ tissues (abdominal lymph nodes, lungs, liver, and spleen) were examined grossly for the presence of tumor. Representative tissue blocks from all of the primary injection sites and all of the organ sites suspicious for metastatic tumor were subsequently processed by 10% formalin fixation, paraffin embedding, and preparation of H&E-stained sections for light microscopic examination.

RESULTS

Identification and Confirmation of Psoriasin Interacting Proteins. We used full-length psoriasin fused to the GAL4 DNA-binding domain as bait in a yeast two-hybrid assay (20) and screened 1.74×10^7 clones from a normal human breast cDNA library. Among 4 true positive clones (26), 1 (Fig. 1*a*) contained almost the full protein sequence (amino acids 42–335) for Jab1. As shown in Fig. 1*a*, controls including unrelated bait (Rad18) and prey (Mad2) constructs, and empty bait and prey vectors did not show any activation of reporter genes. We noted that a Jab1-binding motif common to several Jab1 interacting proteins described recently is also contained within psoriasin (Fig. 1*b*), so we tested whether this region was necessary for the psoriasin-Jab1 interaction. As shown (Fig. 1*a*), only the COOH-terminal portion of psoriasin that contains this motif interacted with Jab1. To additionally confirm the psoriasin-Jab1 interaction in breast cancer cells, psoriasin was stably transfected into MDA-MB-231 cells, and coimmunoprecipitation experiments performed using Jab1 and psoriasin antibodies. Psoriasin-Jab1 protein complexes were detected in both psoriasin-transfected MDA-MB-231 cells (231-HP2) and the breast cell line MDA-MB-468 (which exhibits endogenous psoriasin expression) when Jab1 antibody was used for immunoprecipitation (Fig. 1*c*). However no psoriasin-containing complex was detected in control lanes in the absence of Jab1 antibody or protein G beads. Psoriasin-specific antibody immunoprecipitated psoriasin from cell lysates but was unable to coimmunoprecipitate Jab1 (data not shown), presumably because of the proximity and partial overlap of the epitope recognized by the antibody (amino acids 88–101 of psoriasin) and the proposed Jab1-binding domain (amino acids 57–89 of psoriasin).

Psoriasin and Jab1 Cellular Localization. We localized psoriasin and Jab1 in the MDA-MB-231 clones by immunohistochemistry. Jab1, like psoriasin, has been found previously to be both nuclear and cytoplasmic in cell types other than breast. In MDA-MB-231 cells and all 4 of the transfected cell lines (231-neo, 231-LP1, 231-HP1, and 231-HP2) Jab1 is expressed at comparable levels in the cytoplasmic compartment (Fig. 2, *right panel*). However, in all three of the psoriasin-expressing clones, 231-LP1, 231-HP1, and 231-HP2 (Fig. 2, *left panels*), there is a relative increase in Jab1 within the nucleus. However, the total amount of Jab1 protein as detected by Western blot is similar in all of the cell clones and does not change in the presence of psoriasin (Fig. 3*a*). Psoriasin can also be detected by immunoprecipitation of medium conditioned by 231-HP2 and MDA-MB-468

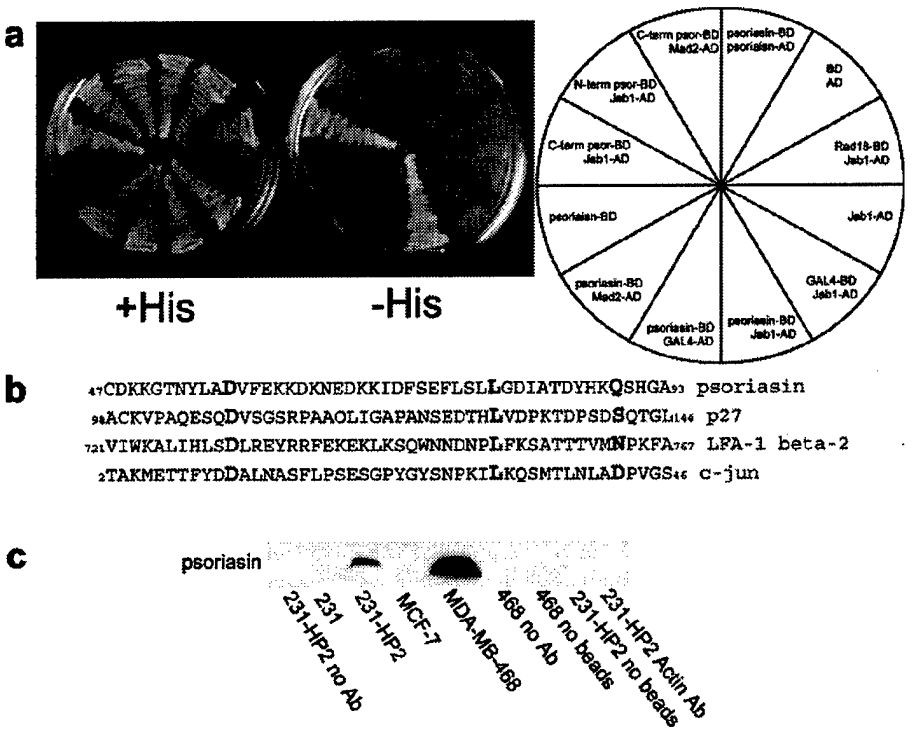


Fig. 1. Psoriasin specifically interacts with Jab1 in yeast two-hybrid assay and breast cancer cells. *a*, the psoriasin-Jab1 interaction was confirmed in yeast, by testing psoriasin fused to the GAL4-BD (binding domain) with selected proteins fused to the GAL4-AD (activating domain). Yeast plates (+ and − Histidine) are shown on the *left*, and the plating schema for each sector is shown on the *right*. Activation of reporter genes and colony growth is only present when the full-length psoriasin or the COOH-terminal half of psoriasin is combined with Jab1. *b*, the COOH-terminal half of psoriasin contains sequences similar to a Jab1-binding domain identified previously in $p27^{Kip1}$, LFA-1, and c-jun. *c*, psoriasin can be coimmunoprecipitated with Jab1 complexes from breast cancer cell lysates. Western blot using antipsoriasin antibody to detect psoriasin, which coimmunoprecipitated with Jab1, from a panel of breast cell lines (see "Materials and Methods").

cells (data not shown), suggesting that psoriasin is also secreted by breast cancer cells in culture.

Psoriasin Overexpression Influences Several Jab1-related Functions. Jab1 influences a number of cellular proteins. Among these, Jab1 effects the level of the negative cell cycle-regulating protein $p27^{Kip1}$ by promoting the export of $p27^{Kip1}$ from the nucleus to the cytoplasm and the subsequent degradation by the Ub-28S proteasome (29). Therefore, we first examined $p27^{Kip1}$ expression in our MDA-MB-231 clones and found that psoriasin-overexpressing clones showed a consistent reduction in levels of $p27^{Kip1}$ relative to wild-type and control cells (Fig. 3*a*).

To determine whether psoriasin influences other Jab1 functions in breast cancer cells we examined AP-1-dependent transcription in the MDA-MB-231 clones using an AP-1-driven luciferase reporter (Fig. 3*b*). AP-1 activity was increased in all 3 of the psoriasin-transfected clones in close proportion to the level of psoriasin expression (Fig. 3*a*). In the high psoriasin-expressing clones (231-HP1 and 231-HP2) there was a 6.5-fold increase in luciferase activity ($P < 0.0001$). These psoriasin-expressing cells showed no difference in total Jab1 levels assessed by Western blot, compared with non psoriasin-expressing controls (Fig. 3*a*). However, the effect on AP-1 activity is consistent with the redistribution and relative increase in nuclear Jab1 protein detected by immunohistochemistry (Fig. 2) and the findings of others (21). Expression of endogenous AP-1-dependent genes was next examined by RT-PCR (Fig. 3*c*). Psoriasin expression is also associated with an increase in mRNA levels of the endogenous AP-1-regulated genes VEGF (30) and MMP13 (31), and this increase is proportional to the levels of psoriasin in the MDA-MB-231 control and transfected cells.

Jab1 also interacts with HIF-1 (32) and enhances its activity. Expression of HIF-1 and the HIF-1-regulated gene *CAIX* (27) was examined by Western blot. Under hypoxic conditions (0.7% O_2), psoriasin-expressing clones showed a marked and higher induction of HIF-1 compared with control cells (Fig. 3*d, top panel*) and a parallel increase in CAIX protein (Fig. 3*d, middle panel*). However, it was noted that CAIX expression was also increased in psoriasin expressing 231-HP1 and 231-HP2 cells under normoxic conditions. The latter observation is consistent with the recent finding that CAIX can also be regulated by AP-1 (33) and indicates that a component of the CAIX induction seen under hypoxic conditions might be attributable to AP-1, given the involvement of AP-1 as well as HIF-1 in the cellular hypoxic response (34, 35).

Psoriasin Overexpression Influences Breast Tumor Progression *in Vitro*. We next looked for a relationship between psoriasin expression and biological end points relevant to tumor progression in breast cancer cells. The effect of psoriasin on growth of MDA-MB-231 cells was examined and found to be associated with a modest but significant increase in growth rate (Fig. 4*a*) of up to 1.3 fold ($P = 0.0009$). The influence of psoriasin on cellular adhesion, an important parameter of invasion, was measured in an *in vitro* assay. We observed a consistent reduction in cell-substrate adhesion (Fig. 4*b*) in psoriasin-expressing clones plated on plastic (0.42-fold reduction; $P < 0.0001$), collagen I (0.20-fold reduction; $P < 0.0001$), and fibronectin (0.18-fold reduction; $P < 0.0001$). The influence of psoriasin on invasion was then assessed in a modified Boyden chamber assay. There was a 1.4-fold increase in invasiveness in the high psoriasin-expressing clones ($P < 0.0001$) after 12 h (Fig. 4*c*), at which time there was no significant difference in growth (data not shown).

Psoriasin Overexpression Influences Breast Tumor Progression *in Vivo*. To determine whether psoriasin expression can also influence invasion and metastasis *in vivo*, psoriasin-overexpressing cells (231-LP1 and 231-HP1) and control cells (parental 231 and 231-neo) were injected into the mammary fat pad of nude mice, and the generation of tumors and metastasis was assessed (Fig. 5). Control cell lines (231 and 231-neo) generated tumors in 2 of 10 and 3 of 10 sites, respectively, after 8 weeks. These tumors were first noted between 2 and 3 weeks after injection, and increased slowly in size (Fig. 5*a*). Both psoriasin-expressing cell lines (LP1 and HP1) generated grossly detectable tumors in 7 of 10 and 6 of 10 sites. These tumors were also first noted between 2 and 4 weeks after injection but

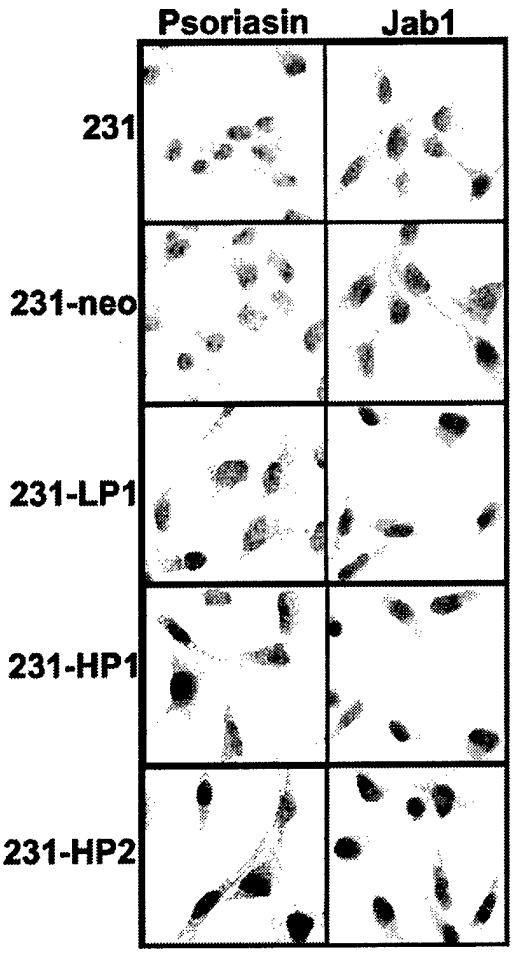


Fig. 2. Psoriasin expression is associated with redistribution of Jab1 to the nucleus. Psoriasin and Jab1 were detected in cells by immunohistochemistry, and representative fields of each cell line are shown. *Left panels* show lack of psoriasin expression in control MDA-MB-231 cells and 231-neo cells, and the expression in the psoriasin transfected clones (231-LP1, 231-HP1, and 231-HP2). *Right panels* show comparable cytoplasmic Jab1 expression in each of the corresponding cell lines, but with enhanced nuclear Jab1 in all three psoriasin overexpressing clones.

increased rapidly in size (Fig. 5, *b* and *c*). By week 8 there was no difference in incidence or mean tumor size between parental 231 cells and 231-neo controls, or between the two psoriasin-expressing clones (Fig. 5*e*). However, both psoriasin-expressing clones were significantly different from both parental and neo-transfected control cells ($P = 0.017$ and $P = 0.024$, Mann Whitney; Fig. 5*f*). Overall mean^{SD} tumor sizes (mm^3) for each experimental group were; MDA-231 = 21^{11}, 231-neo = 54^{8}, LP1 = 336^{223}, and HP1 = 370^{270}. When control groups and psoriasin transfectant groups were combined, the mean^{SD} tumor sizes (mm^3) were also significantly different: MDA-213 + 231-neo = 40^{20} and LP1 + HP1 = 352^{236} combined ($P = 0.0016$, Mann Whitney test). Microscopic examination of primary injection sites identified one additional microscopic tumor in the LP1 cell line group. The primary tumors derived from both control and psoriasin-expressing cells showed similar histological appearances. Expression of psoriasin was confirmed in representative tumors derived from psoriasin-transfected cell clones by immunohistochemistry (data not shown) and by Western blot (Fig. 5*d*). Psoriasin expression was only detected in tumors from psoriasin-transfected cells (although only a very weak signal was detected in the LP1 cell line). p27 expression was reduced in both psoriasin-transfected cell clone tumors. Grossly evident metastasis was identified and confirmed by microscopy in abdominal lymph nodes distant from the primary injection sites in 2 of 10 mice injected with psoriasin-overexpressing cells (both in the HP1 cell line group) compared with 0 of 10 mice in the control experimental groups.

DISCUSSION

The transition from normal epithelium through DCIS to invasive breast cancer is likely to involve many complex processes that are influenced by dynamic changes in gene expression (36). Perhaps the most critical of these processes is the acquisition of the invasive phenotype (37) that occurs with the transition from DCIS to invasive disease, because this event transforms an otherwise local disease into one that is capable of distant spread to threaten the host. It is likely that some of those genes that show alterations in expression between preinvasive and invasive components of breast tissues may be relevant to the process of invasion and offer markers of risk of early tumor progression (36). In this study we demonstrate that the psoriasin gene,

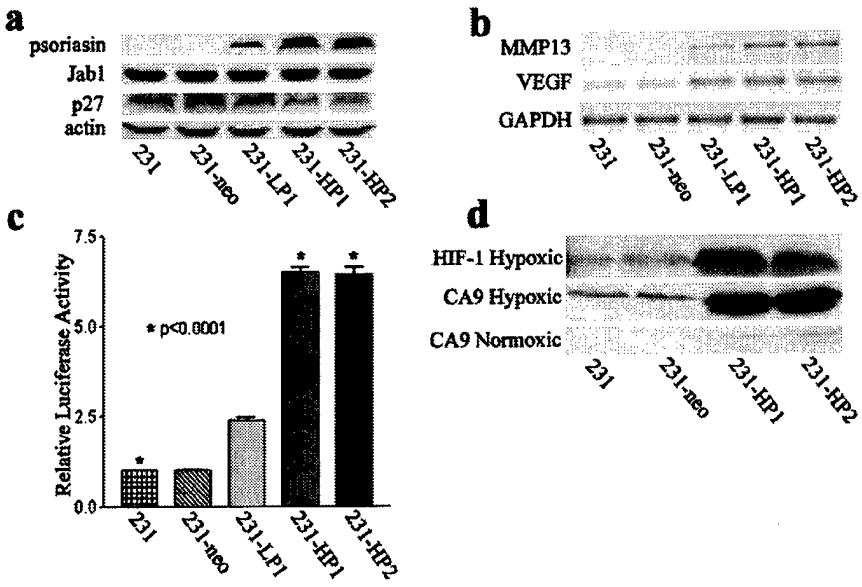
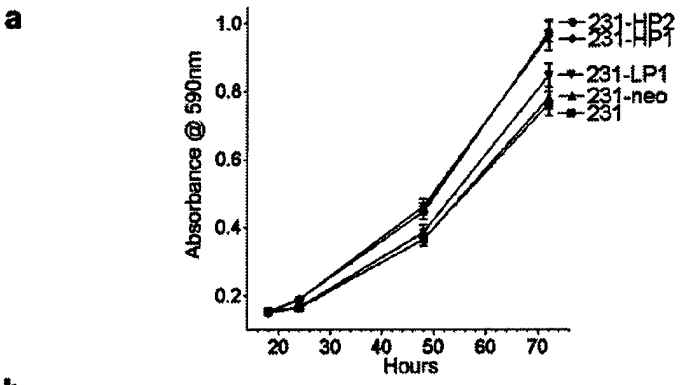


Fig. 3. The biological effects of psoriasin are mediated through Jab1. *a*, Western blot showing absent psoriasin expression in MDA-MB-231 and control 231-neo cells, and the relative levels of psoriasin expression in 3 transfected clones (231-LP1, 231-HP1, and 231-HP2). The total amount of cellular $p27^{Kip1}$ is reduced in psoriasin-expressing clones, whereas the total amount of Jab1 does not change. Actin is shown as a loading control. *b*, psoriasin is associated with an increase in AP-1 activity, as tested by transfection of an AP-1-driven luciferase reporter plasmid. The relative increase in luciferase activity is proportional to the level of psoriasin in the MDA-MB-231 control and transfected cells. *c*, RT-PCR assay to show that psoriasin is also associated with an increase in mRNA levels of the endogenous AP-1-regulated genes VEGF and MMP13 that is also proportional to the levels of psoriasin in the MDA-MB-231 control and transfected cells. *d*, Western blot to shown that psoriasin expression is associated with up-regulation of HIF-1 and induction of the HIF-1-regulated gene, CAIX. Under normoxic conditions there is an increase in CAIX expression in psoriasin-expressing clones. Under hypoxic conditions there is a marked increase in induction of both HIF-1 and CAIX in the psoriasin-expressing clones; *bars*, ±SD.



b

Table 1- The Influence of Psoriasin on Cellular Adhesion to Selected Physical Surfaces

	Plastic	p-value	Collagen I	p-value	Fibronectin	p-value
231	1.000	-	1.000	-	1.000	-
231-neo	1.015 +/- 0.101	ns	1.008 +/- 0.065	ns	1.005 +/- 0.058	ns
231-LP1	0.752 +/- 0.089	p<0.0001	0.935 +/- 0.077	p=0.0027	0.941 +/- 0.067	p=0.0017
231-HP1	0.574 +/- 0.074	p<0.0001	0.802 +/- 0.076	p<0.0001	0.826 +/- 0.066	p<0.0001
231-HP2	0.591 +/- 0.080	p<0.0001	0.792 +/- 0.076	p<0.0001	0.819 +/- 0.065	p<0.0001

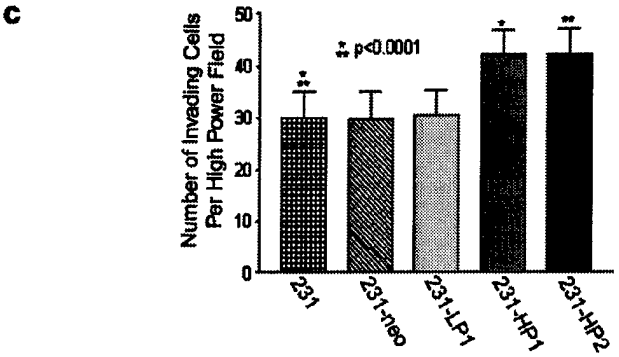


Fig. 4. Psoriasin expression alters cellular features associated with increasing malignancy in breast cancer. *a*, graph to show the growth rate of control cells (MDA-MB-231 and 231-neo) and relative increased growth of psoriasin-expressing clones (231-LP1, 231-HP1, and 231-HP2). Lines and data points represent the means of three independent experiments, with 12 data points at each time point within each experiment. *b*, cellular adhesion to plastic-, collagen I-, and fibronectin-coated surfaces is decreased significantly by psoriasin expression. Numbers represent relative adhesion to MDA-MB-231 cells on each substrate, and data represents the means of three independent experiments, with 12 data points for each cell line on each substrate. *c*, graph to show increased invasiveness in psoriasin-overexpressing cells in the *in vitro* Boyden chamber assay, which parallels the relative decrease in adhesion. *Columns* and *bars* represent means and SDs of three independent experiments where each cell line was assayed in triplicate.

which is highly expressed in DCIS and associated with poor prognostic factors when expressed in invasive disease, can enhance growth, adhesion, and invasiveness of a breast cell line in *in vitro* assays and tumorigenicity in nude mice *in vivo*. Furthermore, we describe a potential mechanism for these effects through a direct interaction between psoriasin and the multifunctional intracellular protein Jab1 (21).

Jab1 was originally identified in mammalian cells as a factor influencing c-jun transcription of AP-1-regulated genes (21). It soon became clear that Jab1 was also a component (CSN5) of a multimeric protein complex (22, 23). The CSN/COP9 signalosome had been studied previously in other systems and shown to be involved in protein degradation via the Ub-26S proteasome (24, 25). Jab1 has since been shown to be involved in a diversity of interactions with components of cell signaling pathways in *in vitro*, yeast, and human cell line model systems. These interactions appear to result in either translocation of Jab1 from cytoplasm to nucleus (integrin LFA-1 [38], erbB-2 [39] signaling), enhanced activity of transcription factors (including c-jun/AP-1 [21], HIF-1 [32], steroid receptors and cofactors [40, 41]) or the promotion of degradation of the interacting protein (including Smad4 [42], p53 [43], HIF-1 [32], MIF1 [28], and $p27^{Kip1}$ [29, 43]), often but not always associated with translocation from nucleus to cytoplasm. However, the physiological relevance of some of these interactions, and specifically in the context of breast epithelial cells, is mostly unknown.

In ovarian tumors, increased nuclear Jab1 is associated with progression and poor outcome (44), and altered Jab1 has also been implicated in renal cancer (45). A direct role for Jab1 in breast cancer has not been identified previously; however, several proteins including p53 and erbB-2, which are known to interact with or to influence Jab1, are altered at an early stage within high-risk DCIS (46–49) and may exert some of their effects through Jab1. The interaction between psoriasin and Jab1 also has the potential to directly facilitate several aspects of early tumor progression. We have shown here that overexpression of psoriasin is associated with translocation of Jab1 to the nucleus, alterations in expression of several Jab1 "downstream" genes, and increased proliferation, altered response to hypoxia, and promotion of invasion. Increased proliferation may be specifically attributable to increased AP-1 activity and down-regulation of the cell cycle inhibitor $p27^{Kip1}$ in this model. Alteration of Jab1 might also lead to increased activation of estrogen receptor and progesterone receptor, and up-regulation of cyclin D1 and alteration of transforming growth factor β signaling in other cell models (39, 50, 51), but these aspects of Jab1 function remain to be examined in the context of breast cancer. Increased capacity to survive hypoxic stress may occur through augmented HIF-1 activity and hypoxic response. Increased invasiveness may result from activation of AP-1 and HIF-1-dependent genes (52, 53), such as matrix metalloproteinases and VEGF, which are already implicated as critical factors in breast tumor progression (37, 54).

The estrogen receptor-negative MDA-MB-231 breast cell line was selected to reflect the context of psoriasin expression that we and others have observed previously in breast tumors *in vivo* (2, 4). The modest although significant increase in proliferation and invasiveness seen in our *in vitro* assays may reflect the fact that this cell line is already a highly proliferative and invasive cell in *in vitro* assay. More

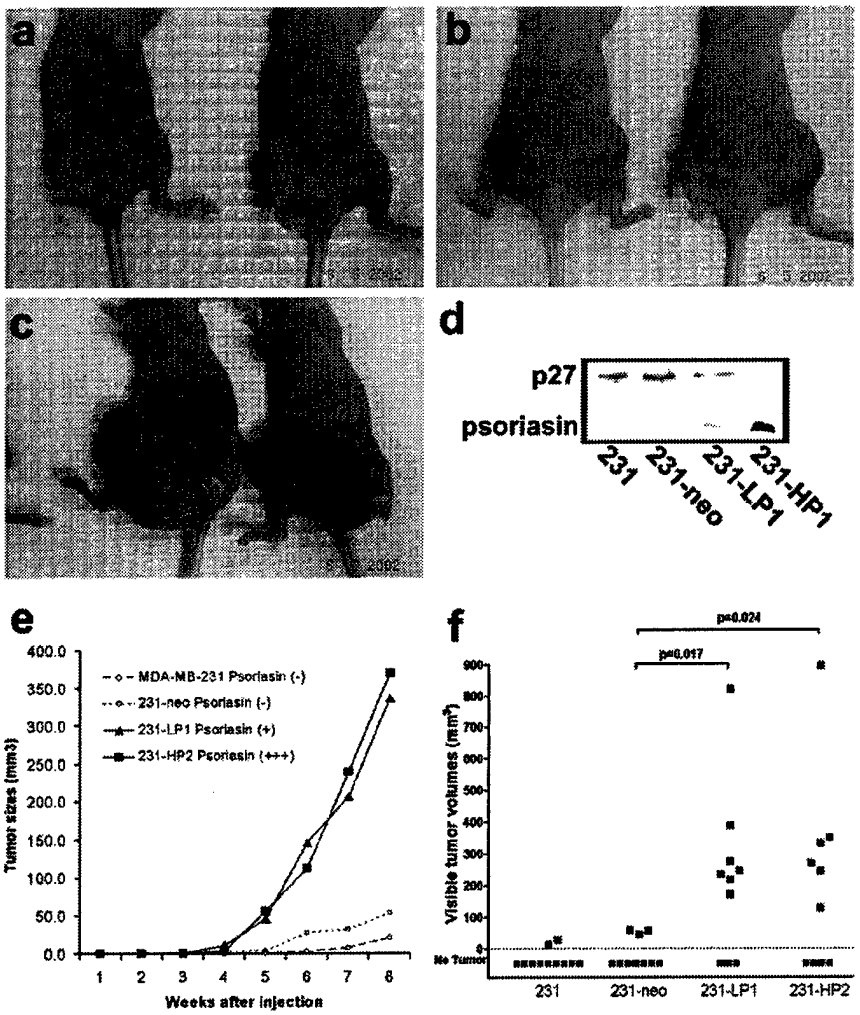


Fig. 5. Effect of psoriasin expression on tumor growth in nude mice. Groups of 5 mice for each cell line received an injection of 5×10^5 cells into the mammary fat pads. The *top panels* (*a–c*) show representative mice at 8 weeks from each treatment group that received (*a*) MDA-MB-231 cells, (*b*) 231-LP1 cells, or (*c*) 231-HP2 cells. *d*, expression of psoriasin and p27 protein determined by Western blot on extracts from representative tumors. *e*, relative growth curves for mice in each group. *Lines* and *data points* indicate the mean tumor volumes at each time point. *f*, distribution of tumor volumes at 8 weeks. Statistical significance was determined by Mann-Whitney test.

striking increases in growth and invasiveness were observed *in vivo* in the nude mice experiments, where metastasis was also associated with psoriasin-expressing tumors. This difference is consistent with the anticipated effects of enhanced metalloproteinase and VEGF expression on extracellular matrix and angiogenesis, spheres of influence that are not adequately replicated in *in vitro* assays, and has been observed by others studying the effects of overexpression of VEGF in breast cell lines (55). Nevertheless, additional detailed studies will be necessary to confirm the direct relationship and functional role of these specific factors in the enhanced growth and invasiveness seen in this model *in vivo*.

Alteration of Jab1 activity in tumors could be attributable in part to alterations in either the cytoplasmic-nuclear distribution (Refs. 38, 45, 56; as appears to be the case for the effect of psoriasin), the ratio of free Jab1:COP9-associated Jab1 (56), competition between different interacting proteins (42), or direct elevation of Jab1 expression and activation. The relevance of these potential mechanisms of action to breast cancer remains to be resolved, both for psoriasin and several other Jab1-interacting proteins. Nevertheless, it has been demonstrated that the many important activities of Jab1 can be influenced by competition between different interacting proteins (42). For example, p53 can compete with and down-regulate Jab1 activation of c-jun (57), and inhibition of Jab1 causes reciprocal up-regulation of p53 (42) and down-regulation of c-jun in HeLa cells (57). It is also interesting to note that the chemokine MIF can exert the opposite effect on Jab1 to psoriasin (28) with respect to modulation of AP-1 activity and $p27^{Kip1}$ expression. This raises the question of whether these different chemokine molecules might compete to modulate Jab1 activity.

Whereas our data support the involvement of Jab1 in mediating many of the biological actions of psoriasin, additional experiments will be needed to confirm that a direct interaction occurs between the putative Jab1 binding motif (29) on psoriasin and the Jab1 protein, and that direct alterations of Jab1 indeed exert effects on these specific target genes and pathways. It is also possible that some of the functions of psoriasin are mediated through other pathways (58). For example, it has been shown that other secreted S100 proteins (S100B and S100A12) can bind to and stimulate the receptor for advanced glycation end products, leading to activation of intracellular signaling pathways including up-regulation of ras, mitogen-activating protein kinase and nuclear factor $\kappa\beta$ in immune cells (59, 60). Expression of receptor for advanced glycation end products is also associated with invasion in gastric carcinoma (61) and is functionally involved in metastasis (62). Unlike some other S100s with chemokine activities such as S100A9 and S100A12, which are expressed by both epithelial and stromal inflammatory cells (63), expression of S100A7 (psoriasin) is restricted to epithelium, at least in skin and breast. However,

psoriasin is also secreted and could potentially interact with cell surface receptors on immune or epithelial cells.

In summary, we have shown that psoriasin can contribute to breast tumor progression and that its action may be mediated, at least in part, through Jab1. Although other important cellular proteins also influence and may compete for Jab1, psoriasin is one of the most abundant proteins in high-risk DCIS (2) and is likely to exert an important effect on Jab1 activity in breast tumor cells at an early stage of tumor progression. Thus, therapies aimed at modulating the effect of psoriasin may have important potential in the treatment of early breast cancer.

ACKNOWLEDGMENTS

We thank Caroline Leygue for generation of the psoriasin expressing clones, Kate Hole for assisting with the two-hybrid screen, Dr. Jaromir Pastorek for antibody to CAIX, and Dr. Adrian L. Harris for the HIF-1 antibody.

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Appendix 7 **J Mol. Endocrinol. 2003. 30: 13-29**

Putative functional characteristics of human estrogen receptor-beta isoforms

B Peng, B Lu, E Leygue and **L C Murphy**

Department of Biochemistry and Medical Genetics and the Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Canada R3E 0V9

(Requests for offprints should be addressed to L C Murphy; Email: lcmurph@cc.umanitoba.ca)

Abstract

Estrogen receptors (ERα and ERβ) are clearly multifaceted in terms of structure and function. Several relatively abundant ERβ isoforms have been identified, which can be differentially expressed in various tissues. In order to provide insight into the possible role of the ERβ family in breast tissue a study of the putative functions of the human (h) ERβ1, hERβ2 and hERβ5 isoforms was undertaken. Only hERβ1 was found to bind ligand, which induced conformational changes as determined by protease digestion assays. All ERβ isoforms could bind to and bend DNA although the relative efficiency with which they bound DNA differed with hERα>hERβ1>hERβ2>>hERβ5. All ERβ isoforms inhibited ERα transcriptional activity on an estrogen-response element (ERE)-reporter gene. The relative activities were hERβ1>hERβ2 >hERβ5; however, only hERβ1 had transcriptional activity of its own. Both LY117018–hERα and LY117018–hERβ1 complexes alone could activate transcription on a TGF-β3-CAT gene. Although hERβ2 and hERβ5 had no activity alone, they inhibited ERα but not hERβ1 transcriptional activity of transforming growth factor (TGF)-β3-CAT. In marked contrast to activity on an ERE-CAT reporter gene, hERβ1 did not modulate ERα transcriptional activity on a TGF-β3-CAT reporter gene. These data support promoter-specific differential activities of hERβ isoforms with respect to models of ERα regulated gene expression, and suggest that they may have a role in differentially modulating estrogen action.

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Introduction

The estrogen receptor family of steroid hormone receptors is clearly multifaceted (Hall *et al.* 2001) and more complex than originally thought. There are two genes which encode estrogen receptors (ER), ERα and ERβ. Both are ligand regulated transcription factors which classically modulate target gene transcription by binding as homo- and/or heterodimers to estrogen responsive sequences in target gene promoters (Cowley *et al.* 1997). These receptors likely have distinct roles in estrogen action, independent of each other when they are expressed separately (Couse & Korach 1999) but can also have direct interactions due to heterodimerization when the receptors are expressed together in the same target cell (Enmark *et al.* 1997). In addition, both ERs may encode variant isoforms generated by alternative splicing mechanisms (Lu *et al.* 1998, Moore *et al.* 1998). In particular there are data to support variant isoforms of ERβ at the protein level (Fuqua *et al.* 1999, Fujimura *et al.* 2001). Furthermore, we have shown in human breast tissues that variant forms of ERβ are more abundant than the wild-type at least at the RNA level (Leygue *et al.* 1999).

Human (h) ERβ2 (also called hERβcx (Ogawa *et al.* 1998*b*)) and ERβ5 variant mRNAs are missing the wild-type exon 8 sequences and contain extra sequences which are distinct from each other, followed by sequences that are then identical with each other (see Fig. 1). They are predicted to encode C-terminally truncated ERβ-like proteins identical to wild-type until amino acid residue 468 (by reference to the long form of hERβ1) (Ogawa *et al.* 1998*a*). After amino acid 468 hERβ2 is predicted to encode 28 novel amino acids, with the full-length protein having a predicted molecular mass of 55·5 kDa. In contrast, after amino acid 468 hERβ5 is predicted to encode only 5 novel

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amino acids with the full-length protein having a predicted molecular mass of 53 kDa.

Although total ERβ expression appears to decrease between normal breast and ER-positive breast tumors (Leygue *et al.* 1998*b*, Roger *et al.* 2001), the relative expression of the variant ERβ isoforms to the wild-type ERβ can also change during breast tumorigenesis, at least at the RNA level (Leygue *et al.* 1999). This suggests that the expression and/or the activity of the ERβ family of receptors changes during breast tumorigenesis and may have a role in this process as well as having a role in the altered estrogen action that occurs during breast tumorigenesis. In order to provide insight into the possible role of the ERβ family in breast tissue we have undertaken a study of the putative functions of the hERβ1, hERβ2 and hERβ5 isoforms.

Materials and methods

Materials

17β-Estradiol (E_2), 4-hydroxytamoxifen (4-OH-TAM) and CAPS (3-cyclohexylamino-1-propanesulfonic acid) were from Sigma Chemical Co. (St Louis, MO, USA). ICI 182,780 was a gift from Dr A E Wakeling (AstraZeneca Pharmaceuticals, Macclesfield, UK). LY117018 was a gift from Eli Lilly Co. (Indianapolis, IN, USA). [^{3}H]17β-Estradiol, [^{14}C]chloramphenicol, and [^{35}S]-methionine were from New England Nuclear (Boston, MA, USA). [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [^{35}S]-ATP were from ICN Pharmaceuticals (Irvine, CA, USA). All cell culture reagents were obtained from GIBCO/BRL (Burlington, Ontario, Canada).

In vitro transcription and translation

In vitro transcription/translation reactions were performed using a coupled transcription/translation system (TnT coupled Reticulocyte Lysate System; Promega, Madison, WI, USA). Reactions were performed according to the manufacturer's instructions.

Scatchard analysis

Human ERα (pcDNA3·1/wild-type human ERα from HEGO (Green *et al.* 1986)), human ERβ1 (pcDNA3·1 hERβ1, long form of 530 amino acids (Leygue *et al.* 1998*a*, Ogawa *et al.* 1998*a*)), human ERβ2 (pcDNA3·1 hERβ2, long form (Ogawa *et al.* 1998*b*)) and human ERβ5 (pcDNA3·1 hERβ5, long form) proteins were synthesized by *in vitro* transcription–translation as described above. Ligand binding studies were conducted as previously described (Lu *et al.* 2000). *In vitro*-generated receptor was diluted 10-fold in buffer (10 mM Tris-HCl, pH 7·5, 1·5 mM EDTA, 10 mg/ml BSA, 10% glycerol) and kept on ice until use. One hundred microliters of the diluted protein were used in each binding reaction that contained varying concentrations of [^{3}H]E_2 (0·01–100 nM), followed by overnight incubation at 4 °C. Non-specific binding was determined by parallel incubations containing a 200-fold excess of unlabeled E_2. Unbound steroid was removed by addition of 500 µl 0·5% charcoal–0·05% dextran in the above dilution buffer for 30 min at 4 °C followed by centrifugation at 10 000 × ***g*** for 10 min at 4 °C. Radioactivity was determined in an aliquot of the supernatant and in aliquots of total [^{3}H]E_2 solutions using a scintillation counter. The ratio of specifically bound/unbound steroid and the concentration of specifically bound steroid were used for Scatchard analysis, from which was determined the equilibrium dissociation constant, K_d.

Limited proteolytic digestion analysis

Conformational studies were performed as described previously (Beekman *et al.* 1993). *In vitro*-synthesized ERs were incubated with agonists (E_2, diethylstibesterol) and antagonists (4-OH-TAM, LY117018) overnight at 4 °C. The liganded receptors were then diluted 1:10 (v/v) in TE buffer, then 20 µl of this ER solution were treated with increased concentrations of trypsin (0·2 to 5 µg) for 20 min at room temperature and stopped by the addition of loading buffer. The samples were boiled and were analyzed directly by SDS-polyacrylamide gel electrophoresis (10% w/v). The gel was dried and the digested bands were visualized by autoradiography.

Electrophoretic mobility gel-shift assay (EMSA)

In vitro-synthesized human ERs were used for EMSA. Typically 1 µl programmed lysates containing equal amounts of each receptor as determined

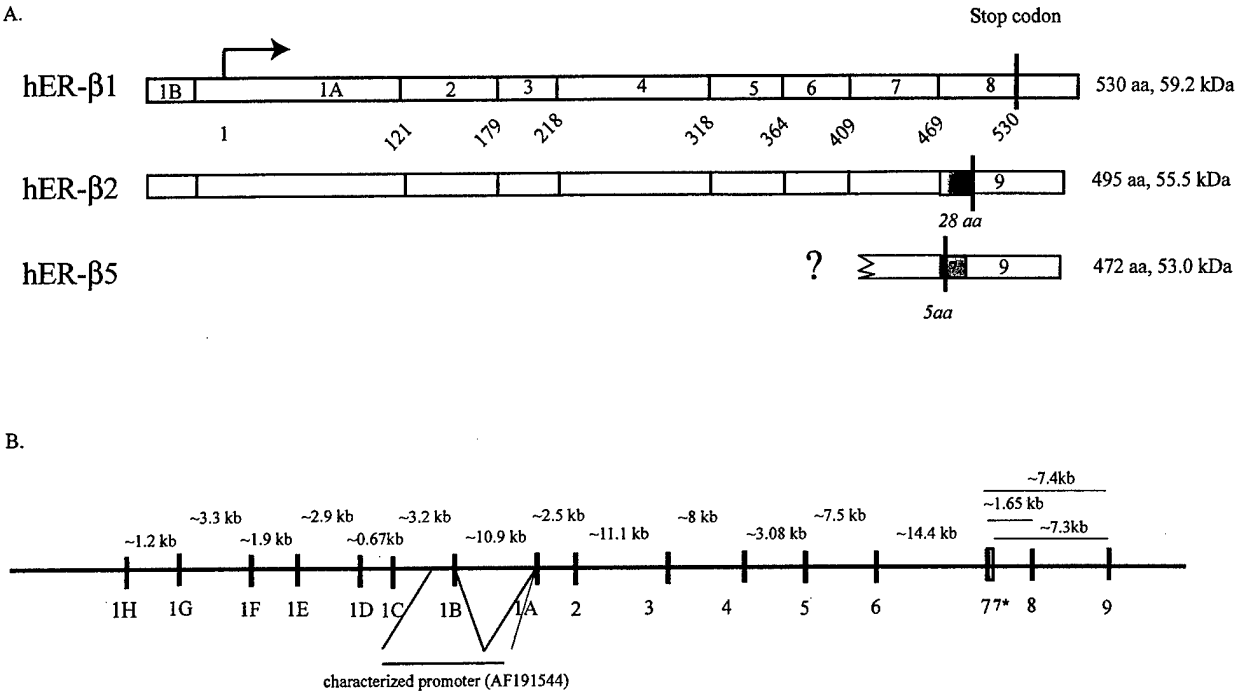


Figure 1 (A) cDNA structure of human ERβ1 (hER-β1), human ERβ2 (hER-β2) and human ERβ5 (hER-β5) and predicted proteins of the human ERβ isoform cDNAs. Genomic structure of the human estrogen receptor β locus on chromosome 14. Human ERβ cDNA, expressed sequence tags and published promoter sequences (accession numbers in the text) were aligned with sequences from two genomic clones of human chromosome 14 (AL162756/CNS01 RHJ and AF215937). Exons 1C to 1H are found only in one cDNA (accession number AB006589). aa, amino acids.

by polyacrylamide gel electrophoresis of ^{35}S-methionine-labeled protein generated in parallel *in vitro* transcription–translation assays, was assayed in EMSA. One microliter lysate was incubated in a final volume of 20 μl, and the reaction solution was 5 mM Tris-HCl, pH 7·5, 100 mM KCl, 2 mM dithiothreitol, 5% v/v glycerol and contained 2 μg polyd(I-C). The binding reaction was initiated by adding 1 μl (approx. 10 fmol) 5′-[^{32}P]-end-labeled, double stranded estrogen-response element (ERE) oligonucleotide (35 mer, 5′-AACTTTGATCAGG TCACTGTGACCTGACTTTGGAC-3′ containing the vitellogenin A2 ERE sequence), and the mix was incubated at 20 °C for 30 min. DNA-bound complexes were electrophoretically separated on a 4·5% loosely cross-linked acrylamide gel (1:29 bis:acrylamide) at 150 V for 90 min at 20 °C in 0·5 × TBE buffer. Gels were then vacuum dried and autoradiographed. To identify immunoreactive ER within retarded DNA-bound complexes, parallel incubations containing 1 μg ER antibody (usually 1 μl H222 for ERα, or 1 μl PAI-310 for ERβ1 and ERβ2) were run to determine the presence of super-shifted antibody-bound ER–ERE complexes (data not shown). Reticulocyte lysates containing *in vitro*-translated ER proteins were incubated with or without saturating concentrations of ligand (estrogen or antiestrogen) at 4 °C overnight to allow receptors to bind ligand, followed by EMSA.

DNA bending assay

The DNA bending vector ERE Bend I (kindly provided by Dr A Nardulli, University of Illinois, Urbana, IL, USA) (Nardulli & Shapiro 1992) was digested with EcoRI and EcoRV to produce a 430 bp DNA fragment with a single consensus ERE either at the end (EcoRI fragment) or in the middle (EcoRV fragment). The fragments were then gel purified, labeled by incubation with polynucleotide kinase in the presence of

[γ-^{32}P]ATP, and purified on a G50 Sephadex column. Gel mobility shift assays were carried out essentially as described above. Aliquots of the binding reactions were run on 8% non-denaturing acrylamide gels, dried and exposed to X-ray film. The degree of DNA bending was determined using the method of Thompson and Landy (1988).

Cells, cell culture and transient transfection

For transient transfection analysis, tagged ER expression vectors were generated. Human ERα, ERβ1, ERβ2 and ERβ5 were tagged at their N-terminus with a polyhistidine and an Xpress epitope tag using the pcDNA4/HisMax(A) plasmid (Invitrogen Canada Inc., Burlington, Ontario, Canada). Cos-1 and Cos-7 cells were obtained from the ATCC (Manassas, VA, USA). The cells were routinely cultured in DMEM containing 5% v/v fetal calf serum (FBS), 1% w/v glucose, glutamine and penicillin–streptomycin (5%CM). To obtain estrogen-depleted cells, the culture medium of stock cells was changed to phenol red-free DMEM supplemented with 5% charcoal dextran-treated FBS, 1% w/v glucose, glutamine and penicillin–streptomycin (5%CS) and replaced every 2 days. Six days later the medium was replaced by 10%CS until required for experiments. For transient transfection experiments, the cells were transfected using the Effectene transfection reagent according to the manufacturer's instruction (QIAGEN, Mississauga, Ontario, Canada). Briefly, the day before transfection, the estrogen-depleted cells were seeded in 6-well plates at $2{\cdot}5 \times 10^5$ cells per well in 2 ml 5%CS and left overnight. The plates were 70–80% confluent on the day of transfection. The transfection mixture was prepared according to the manufacturer's protocol, then fresh medium (5%CS) was added to the transfection mixture and 0·6 ml per well of the above mixture with either ERE-II-TCO-CAT (a gift from P Webb (Webb *et al.* 1995)) or transforming growth factor (TGF)-β3-CAT-reporter plasmid DNA (Yang *et al.* 1996) was added. ER expression plasmid (50–450 ng) or empty vector and 100 ng β-gal pCH110 plasmid DNA (Pharmacia Canada, Mississauga, Ontario, Canada) was added drop-wise into the medium (CS) and the plates were gently swirled to ensure uniform distribution of the DNA–Effectene complexes. Vehicle (ethanol), estradiol-17β or LY117018 was then added 20 to 30 min later. The cells were left for 48 h and then harvested. Cell extracts were prepared by freeze/thawing and were used to determine chloramphenicol acetyl transferase (CAT) and β-galactosidase activity as previously described (Dotzlaw *et al.* 1992).

Western blot analysis

For Western blot analysis, $2{\cdot}5 \times 10^5$ Cos-1 cells were set up in 6-well plates, then transiently transfected with plasmids and treated with estrogen or antiestrogen under the same conditions as for the CAT assay described above. Cells were harvested 48 h after transfection, washed once with Isoton II and then the washed cell pellets were resuspended in 200 μl Isoton II. Aliquots of cell suspension (150 μl) were extracted and used for Western blots and the remainder was used for determination of β-galactosidase activity. For Western blotting, the cells were pelleted and then extracted using 40 μl hot (95 °C) extraction buffer J with shaking for 20 min at 95 °C as previously described by Joel *et al.* (1998). The entire extract was subjected to 10% SDS-polyacrylamide gel electrophoresis as previously described (Adeyinka *et al.* 2002). The separated proteins were transferred to nitrocellulose membranes and processed as previously described (Adeyinka *et al.* 2002). Detection of the tagged estrogen receptor proteins was by incubation of blots with anti-Xpress antibody (1/5000 in TBST, Cat#R910-25, Invitrogen Canada Inc.) overnight at 4 °C, followed by washing and incubation with secondary antibody (horseradish peroxidase conjugated goat anti-mouse antibody, 1/5000 in TBST, Jackson Immuno Research Labs Inc., West Grove, PA, USA) at room temperature for 2 h. Visualization was carried out using the SuperSignal West Dura Extended Duration Substrate kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Statistical analysis

Differences in variance were tested using ANOVA, where appropriate. Differences between individual mean values were then determined using Student's *t*-tests. All tests were performed using GraphPad Prism statistical analysis software (GraphPad Software Inc., San Diego, CA, USA).

Results

Identification and organization of hERβ1, hERβ2 and hERβ5 cDNA sequences at the hERβ locus on chromosome 14

The estrogen receptor beta gene has been localized to human chromosome 14q22–24 and the genomic structure of 8 exons comprising hERβ1 has previously been published (Enmark *et al.* 1997). However, the previously described variant hERβ2 (also called hERβcx) and hERβ5 mRNA contain only sequences corresponding to exons 1 to 7 of hERβ1 and then they diverge (see Fig. 1A). They do not contain exon 8 sequences of hERβ1, but contain sequences termed exon 9, which are located downstream of exon 8 on chromosome 14 (Fig. 1B), identified using database sequences of chromosome 14 (accession numbers CNSO1 RHJ and AF215937) and the Human Genome Working Draft. It should be noted that neither of these genomic sequences contain an extra A 5′ of the start site of translation for hERβ, that would place another upstream ATG in frame with the known coding region and introduce 18 amino acids to the N-terminal of the known coding region, as recently described (Wilkinson *et al.* 2002). Interestingly, hERβ5 mRNA also contains sequences between exon 7 and part of exon 9 which are not present in either hERβ1 or hERβ2 mRNA. These hERβ5 mRNA specific sequences can be found immediately following exon 7 sequences in intron 7 of the human ERβ gene (Fig. 1B), suggesting that the normal splice donor site is not recognized and a cryptic splice donor site is present in intron 7. Furthermore the exon 9 sequences present in hERβ5 cDNA start 28 nucleotides downstream of those present in hERβ2, suggesting a cryptic splice acceptor site is present within exon 9. There are also multiple non-coding exons 5′ to exon 1 as previously identified (Enmark *et al.* 1997), since several hERβ cDNAs contain sequences in their 5′ UTR which are found further upstream of the previously described exon 1 on chromosome 14 (Fig. 1B; 1H–1C seen in AB006589, 1B seen in NM_001437, AX234658, AF05428, AF060555, AB006589 references). The sequences of a recently characterized promoter region of hERβ (Li *et al.* 2000) are found immediately upstream and overlapping with exon 1B. However, the presence of hERβ mRNAs whose 5′UTR contain exonic sequences found upstream of this documented promoter suggest that there are alternative promoters for the hERβ gene. This is similar to the hERα gene and suggests that regulation of expression of these genes is complex (Kos *et al.* 2001).

The predicted open reading frames for hERβ1, hERβ2 and hERβ5 are shown in Fig. 1A. hERβ2 contains amino acids 1–468 which are identical to hERβ1; the sequence then diverges containing another 28 novel amino acids encoded in the open reading frame. hERβ5 was isolated as a partial cDNA but is likely also to be identical to hERβ1 from amino acids 1–468 and then diverges containing another 5 novel amino acids. Both these variant hERβ proteins would be truncated at the C-terminus, disrupted in helix 11 and missing helix 12 and therefore unlikely to bind ligand or have AF2-mediated transcriptional activity. Lack of ligand binding has been confirmed using *in vitro* generation of these proteins as outlined below.

Ligand binding activity of hERβ1 and variant isoforms hERβ2 and hERβ5 proteins

Human ERβ1 has previously been shown (Enmark *et al.* 1997) to bind E_2 with high affinity and specificity, and our data confirm these findings. Figure 2A shows specific saturable binding of [^{3}H]E_2 to *in vitro* translated hERβ1 with a calculated K_d=0·11 nM. However, the open reading frames of hERβ2 and hERβ5 cDNA predict for C-terminally truncated proteins compared with hERβ1 and are predicted not to bind ligand. As shown in Fig. 2B and C no saturable binding of [^{3}H]E_2 to *in vitro*-translated hERβ2 or β5 was observed.

Human estrogen receptor isoform conformational status and ligand induced changes

To determine the possible conformational status of variant hERβ isoforms, a previously used limited trypsin digestion assay (Beekman *et al.* 1993, McDonnell *et al.* 1995) was employed to compare the proteolytic digestion patterns of variant ^{35}S-methionine labeled hERα, hERβ1, hERβ2 and hERβ5 in the presence and absence of estrogens and antiestrogens (4-OH-TAM and LY117018). The results are shown in Fig. 3. In the absence of any ligand, all ER isoforms were sensitive to

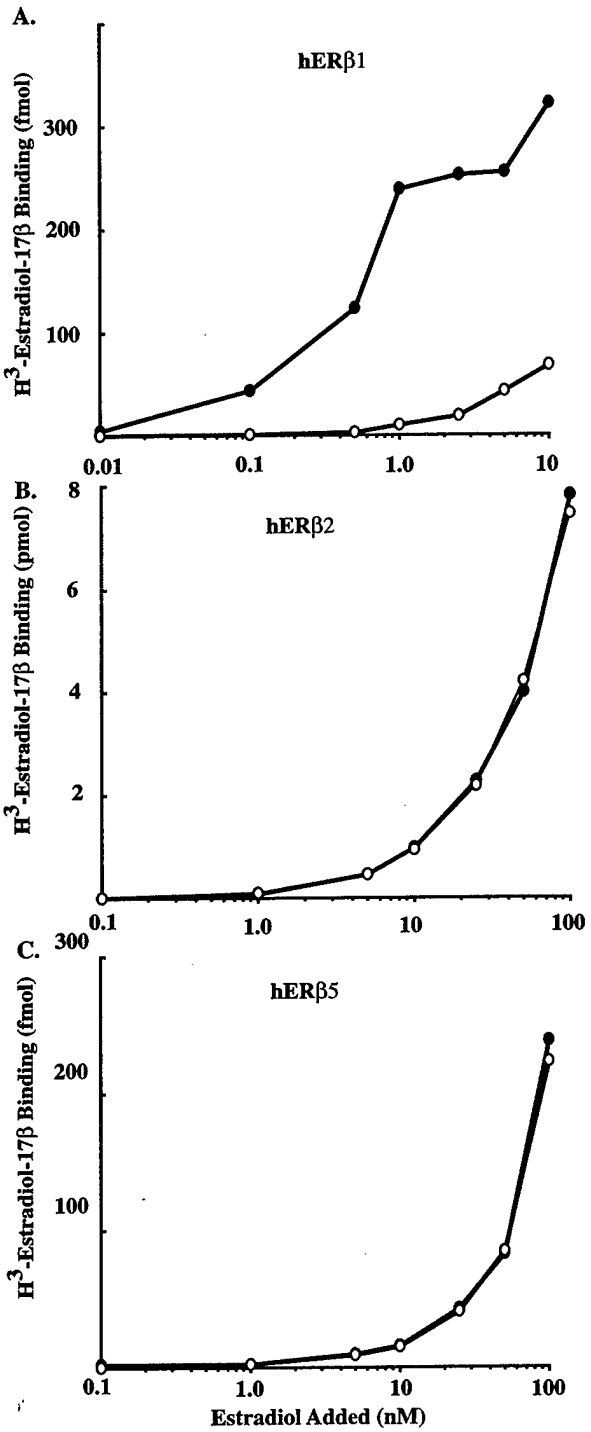


Figure 2 Determination of estradiol binding to (A) hERβ1, (B) hERβ2, and (C) hERβ5. Increasing amounts of [^{3}H]estradiol-17β (0·01–100 nM) were incubated with a constant amount of each *in vitro*-transcribed/translated hERβ protein. Two separate experiments were performed. Total binding is shown by the closed circles and non-specific binding is shown by the open circles.

proteolysis. In the presence of estradiol both hERα and hERβ1 become more resistant to digestion, and a 32·5 kDa resistant band (shown by asterisks in Fig. 3) was observed. In contrast addition of the antiestrogens 4-OH-TAM and LY117018 did not significantly affect the sensitivity of the receptors to trypsin compared with the receptors in the absence of ligand. The sensitivity of the variant isoforms hERβ2 and hERβ5 was not affected by ligand, consistent with their inability to bind ligand and suggesting that the variant isoforms are unlikely to be in an activated conformation.

DNA binding and bending activity of hERβ1 and variant isoforms hERβ2 and hERβ5 proteins

Similar amounts of each recombinantly produced ER isoform protein, determined as described in the Materials and methods section, were used in the electrophoretic mobility shift assays. As previously demonstrated hERβ1 and hERβ2 can bind to an ERE in a gel mobility shift assay (Fig. 4A), although the efficiency of hERβ2 DNA binding was less than hERβ1 (Moore *et al.* 1998). In contrast, Ogawa *et al.* (1998*b*) showed no DNA binding activity for hERβ2. hERβ5 also has the ability to bind an ERE in gel mobility shift assays (Fig. 4A), but was less efficient than hERβ2. The specificity of the binding was determined by competition with excess unlabeled ERE whereas no competition was seen with an excess of unlabeled nonspecific 33 mer oligonucleotide.

DNA bending assays demonstrated that hERα, hERβ1 and hERβ2 were all able to bend DNA as demonstrated by the reduced mobility of complexes when the ERE is in the middle of the DNA fragment (M) in comparison with the mobility of complexes when the ERE is at the end (E) of the DNA fragment (Fig. 4B and C; Nardulli & Shapiro 1993, Lu *et al.* 2000). The calculated bending angle for hERα was 64·8 ± 1 (mean ± S.E.M, n=3), for hERβ1 it was 53·6 ± 0·5 and for hERβ2 it was 54·6 ± 0·7. A lower overall signal of the retarded complexes was seen with hERβ1 and hERβ2 compared with hERα which likely reflects the lower efficiency of the hERβ isoforms of binding to an ERE compared with the hERα. Furthermore, the DNA binding ability of hERβ5 was too low to obtain accurate data for DNA bending calculations. No effect of ligand was observed (data not shown).

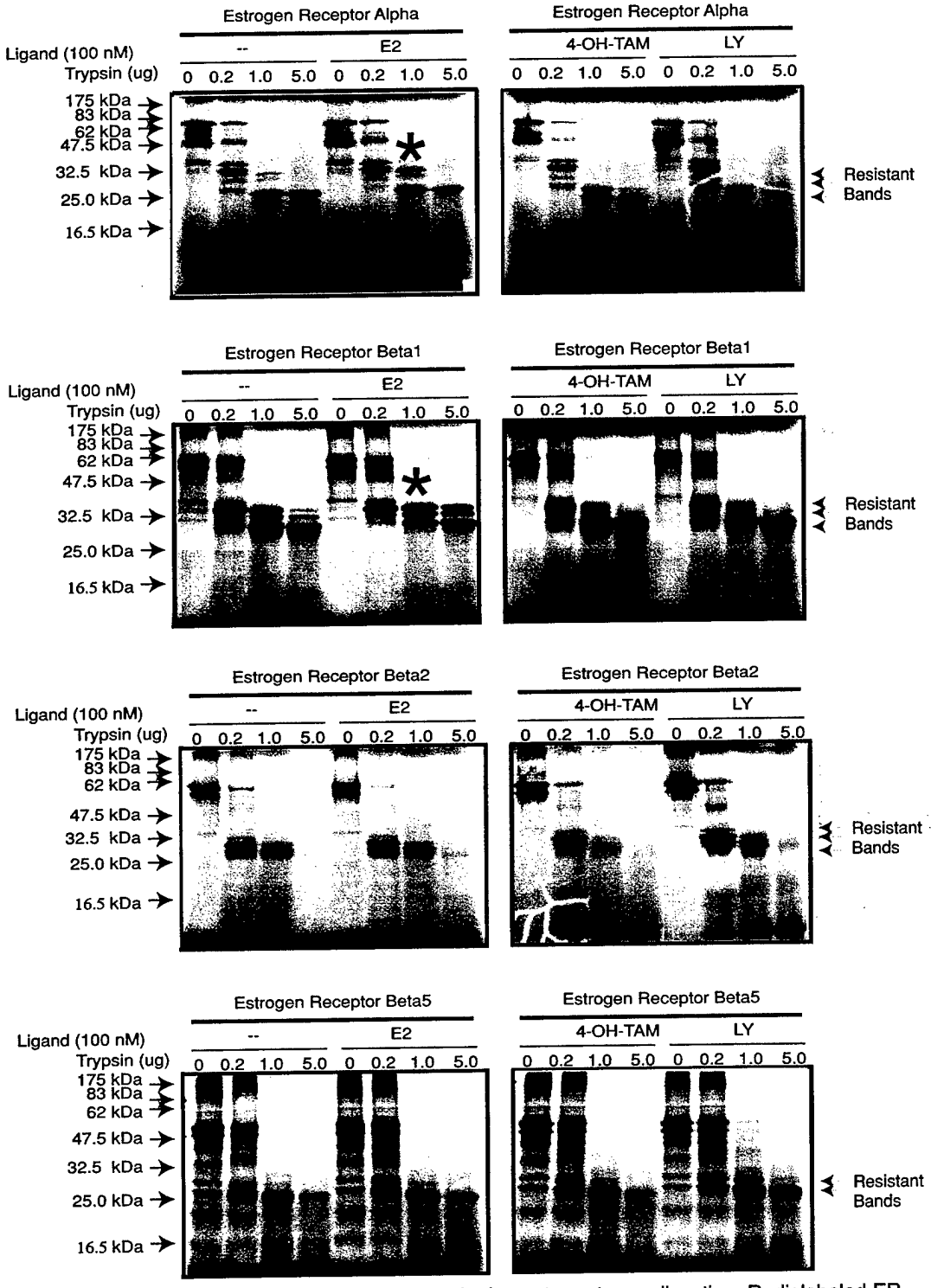
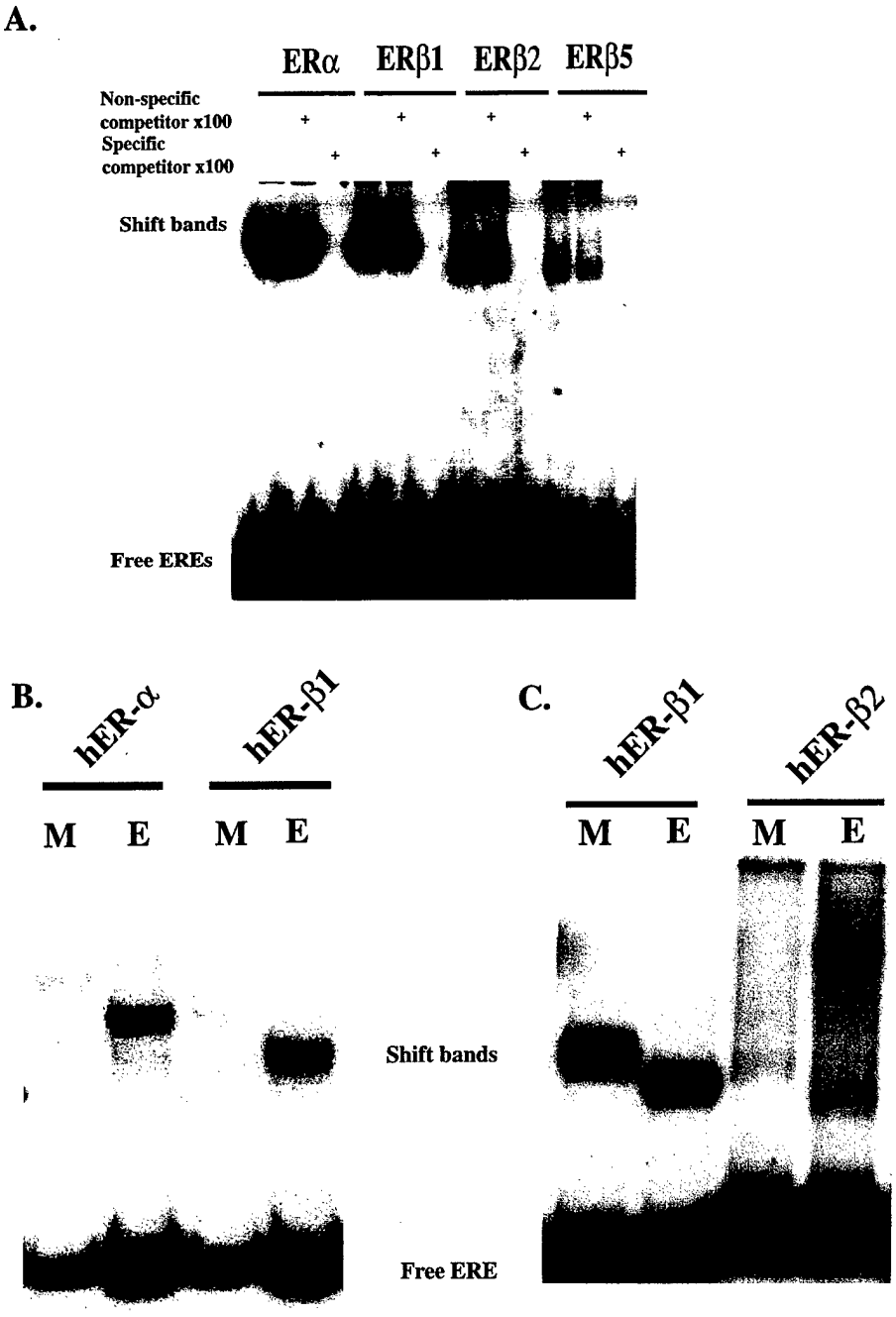


Figure 3 Sensitivity of human estrogen receptor isoforms to protease digestion. Radiolabeled ER was made *in vitro* as described in the Materials and methods section, and digested with increasing levels of trypsin, with and without ligand (E_2, 4-OH-TAM or LY117018 (LY)). The products were visualized by autoradiography after SDS-PAGE. Resistant bands are shown by arrows. The asterisks show the agonist induced resistant 32·5 kDa bands.

Transcriptional activity of hERβ1, hERβ2 and hERβ5

The ability of tagged ERs to activate transcription was initially investigated using Cos-1 cells and an ERE containing reporter gene, ERE_{45}2-delta-TCO-CAT, which has two vitellogenin A2 (−333/−288) EREs upstream of a CAT reporter (Webb *et al.* 1995). Epitope tagged receptors were used so that relative expression of all the ERs could be measured using antibodies to the epitope tag, and preliminary experiments demonstrated that the tagged ERα and ERβ1 were similar to their untagged counterparts in activating transcription with and without ligand (data not shown). Preliminary studies showed that transfection of



50 ng ERα expression vector gave maximal estradiol-induced transactivation of this reporter gene. hERα and hERβ1 activated transcription in a ligand inducible manner (Fig. 5), but hERβ1 was overall less active than hERα ($P<0{\cdot}0001$, $n=5$), and increased expression of hERβ1 did not alter this relationship. These data are consistent with previous findings. As shown in Fig. 5, low doses of estradiol (0·1 nM) which significantly activated hERα did not activate hERβ1 ($P<0{\cdot}0001$, $n=5$), and the apparent ligand-independent activity (zero ligand added) of hERα was significantly higher than that of hERβ1 ($P=0{\cdot}024$, $n=5$). This ligand-independent activity was inhibited by 0·1 and 100 nM of the antiestrogen LY117018 (a raloxifene analog) as well as by hERβ1 (50–450 ng) and hERβ2 (450 ng) (data not shown). These results are not due to over-expression of hERβ1 protein relative to hERα, since under conditions where similar levels of hERα protein (50 ng hERα expression plasmid; see Fig. 6, lane 4) and hERβ1 are expressed (150 ng hERβ1 expression plasmid; see Fig. 6, lane 4) the ligand-independent activity of the receptors is still significantly different (see Fig. 5, compare histogram bar 1 with 7) and the estrogen-inducible (0·1 and 100 nM) activity of hERβ1 (see Fig. 5, compare histogram bars 2 and 3 with histogram bars 8 and 9) is not further increased. The expression of the variant isoforms hERβ2 and hERβ5 alone demonstrated little if any transcriptional activity under these conditions (Fig. 5).

ERα and hERβ isoforms can heterodimerize (Cowley *et al.* 1997), which may underlie the functional interactions between ER isoforms. All hERβ isoforms tested inhibited the transcriptional activity of hERα on an ERE containing promoter (Fig. 7A and B) but the various hERβ isoforms had different efficiencies with hERβ1>hERβ2>hERβ5. Ligand activation of hERβ1 did not affect its ability to decrease the activity of hERα, since under conditions when it was not activated (0 or 0·1 nM estradiol, see Fig. 5) hERβ1 activity was similar to that under conditions when it was activated (100 nM estradiol). Variant isoforms of hERβ had little if any effect on hERβ1 activity on ERE-containing promoters (data not shown).

The transcriptional activity of ER isoforms was next examined on the non-ERE-containing promoter, TGFβ-3-CAT, where the DNA binding domain of ERα is not required for activity (Yang *et al.* 1996). This promoter was shown to be preferentially activated by the raloxifene-bound hERα compared with estradiol in cultured cells (Yang *et al.* 1996), and we have previously shown differential abilities of murine ERβ isoforms to affect this promoter compared with ERE-containing promoters (Lu *et al.* 2000). Therefore, the activity of hERβ isoforms on TGFβ-3-CAT was examined (Fig. 8). Optimal activity for hERα was obtained with transfection of 50 ng expression plasmid (data not shown). A significant increase in transcription was obtained with 0·1 nM LY117018 that was not further increased with 100 nM LY117018 treatment ($P=0{\cdot}0061$, $n=3$). LY117018 significantly increased the transcriptional activity of hERβ1 on the TGFβ-3-CAT reporter gene at the lower levels of hERβ1 expression (50 ng, $P=0{\cdot}008$; 150 ng, $P=0{\cdot}02$, $n=3$) but at high levels of hERβ1 expression (450 ng), a significant increase in ligand-independent activity was seen, and no further increase was seen due to ligand. Overall, hERβ1 was significantly less active than hERα in inducing TGFβ-3-CAT ($P<0{\cdot}0001$, $n=3$).

Figure 4 (A) Determination of the ability of hERα (ERα), hERβ1 (ERβ1), hERβ2 (ERβ2) and hERβ5 (ERβ5) to bind to DNA. Autoradiograph of an electrophoretic mobility gel shift analysis of *in vitro*-transcribed/translated hERα, hERβ1, hERβ2 and hERβ5 proteins binding to a 35 mer double stranded ERE oligonucleotide containing the vitellogenin A2 ERE sequence. Free ERE and the shifted complexes are indicated. The presence of the appropriate ER isoform in the shifted complex was determined by the ability of a specific antibody (H222 for ERα, PA1 for the ERβ proteins) to super-shift the complex (data not shown). Specificity of the complexes was determined by the ability of a 200-fold excess of the unlabeled ERE (specific competitor) to compete for the shifted complex and non-specific interactions were determined using a 200-fold excess of unlabeled nonspecific 33 mer oligonucleotide (nonspecific competitor). (B and C) Comparison of the ability of (B) hERα (hER-α) and hERβ1 (hER-β1) and (C) hERβ1 and hERβ2 (hER-β2) to bend DNA. *In vitro*-transcribed/translated ER isoforms were preincubated with 10 nM estradiol-17β followed by incubation with radiolabeled ERE Bend fragments as described in the Materials and methods and were subjected to electrophoretic gel mobility shift analysis. DNA bending was demonstrated by the reduced mobility of complexes when the ERE is in the middle of the DNA fragment (M) in comparison to the mobility of complexes when the ERE is at the end of the DNA fragment (E).

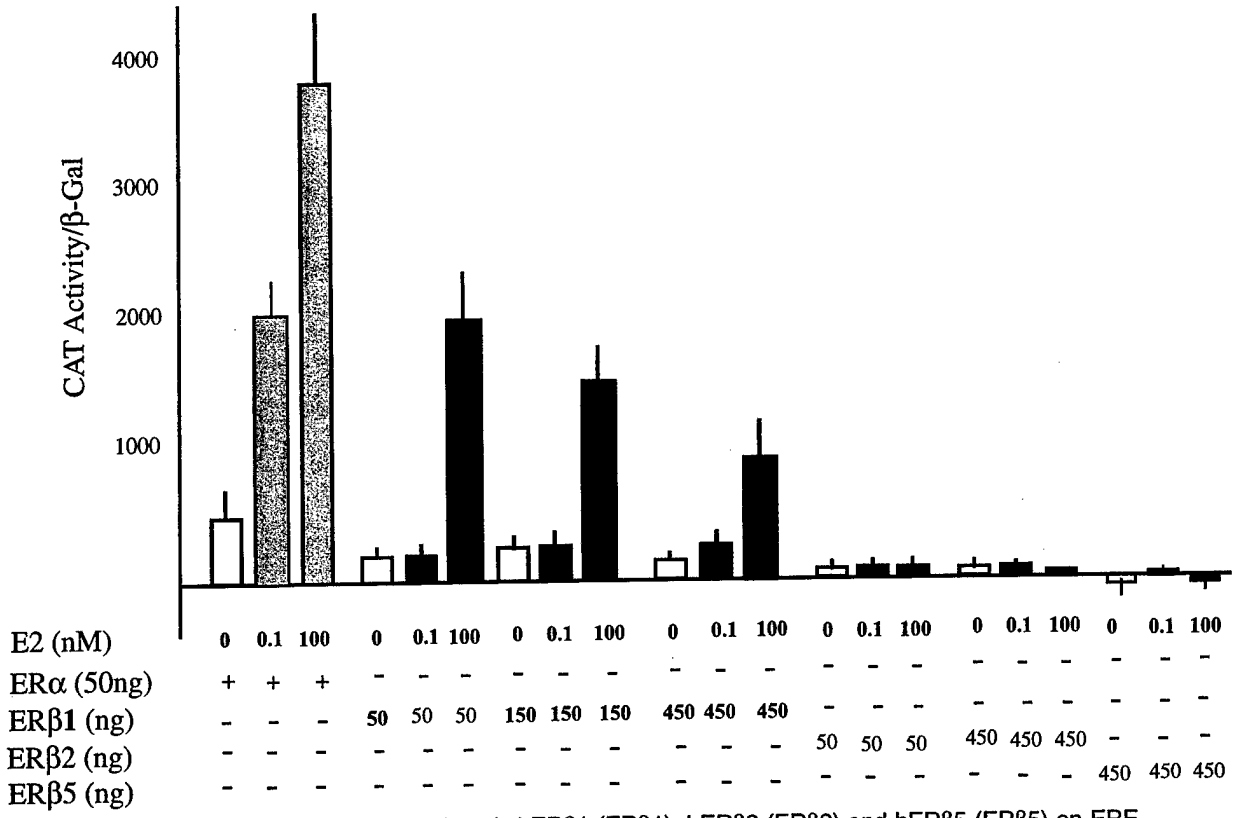


Figure 5 Transcriptional activity of hERα (ERα), hERβ1 (ERβ1), hERβ2 (ERβ2) and hERβ5 (ERβ5) on ERE regulated CAT reporter gene. Increasing amounts of hER isoform expression vectors were co-transfected with an ERE-CAT reporter gene into Cos-1 cells. The transfected cells were treated for 48 h with or without estradiol-17β. Results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency) ± S.E.M. of 5 independent experiments for hERα, hERβ1 and hERβ2, and 3 independent experiments for hERβ5. See text for statistical analysis.

Although there was a trend towards inhibition of TGFβ-3-CAT with increasing expression of hERβ2 or hERβ5 (data not shown), this was not statistically significant.

When the ability of hERβ isoforms to affect hERα activity was investigated at the TGF-β3 promoter, differences between the wild-type and variant isoforms were observed. The wild-type hERβ1 did not significantly affect hERα transcriptional activity at any level of expression tested (Fig. 9A and B). However, under the same conditions hERβ2 significantly inhibited hERα transcriptional activity on TGFβ-3-CAT ($P=0{\cdot}0002$, $n=3$), and as expected the effect was not influenced by LY117018, since hERβ2 does not bind ligand. However, hERβ2 inhibits both the ligand activated and the non-ligand activated (data not shown) hERα ($P=0{\cdot}017$, $n=3$) at the TGFβ-3-CAT promoter. hERβ5 also inhibited hERα transcriptional activity on TGFβ-3-CAT but only at the highest expression of hERβ5 (Fig. 9B, $P=0{\cdot}038$, $n=3$). Similar to their action at an ERE-containing promoter, the truncated ERβ variants ERβ2 and ERβ5 do not modulate wild-type hERβ1 transcriptional activity on TGFβ-3-CAT (data not shown).

Discussion

There is a growing body of evidence that ERα and ERβ can be expressed together in some cell types and independently expressed in others (Dotzlaw *et al.* 1997, Jarvinen *et al.* 2000, Saji *et al.* 2000). If expressed together they form heterodimers, which under experimental conditions are preferred over homodimerization (Cowley *et al.* 1997). Further, transient coexpression of ERα and ERβ in cell lines results in ERβ1-induced reduction of ERα activity

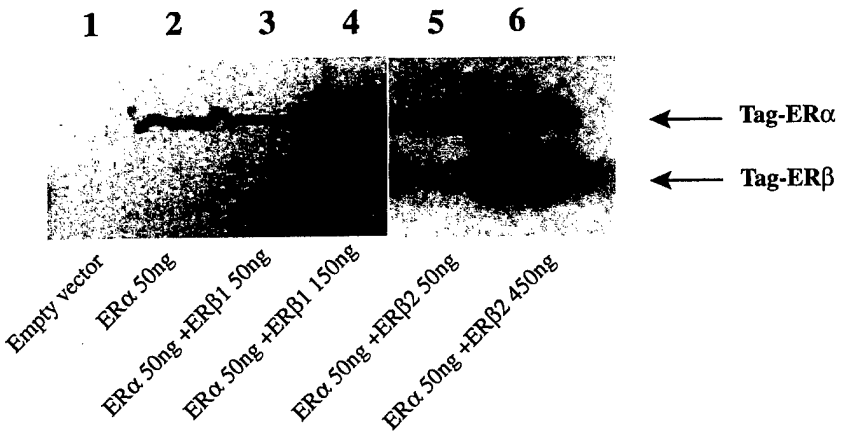


Figure 6 Western blot analysis of Cos-1 cell extracts 48 h after transfection of the indicated amounts of tagged-hERα (ERα), -hERβ1 (ERβ1) and -hERβ2 (ERβ2) expression vectors, as detailed in Materials and methods. Lane 1, extract of Cos-1 cells transfected with empty expression vector alone; lane 2, tagged-hERα expression vector; lane 3, tagged-hERα (50 ng) + tagged-hERβ1 (50 ng) expression vectors; lane 4, tagged-hERα (50 ng) + tagged-hERβ1 (150 ng) expression vector; lane 5, tagged-hERα (50 ng) + tagged-hERβ2 (50 ng) expression vectors; lane 6, tagged-hERα (50 ng) + tagged-hERβ2 (450 ng) expression vectors. The tagged proteins were visualized with anti-Xpress antibody as described in Materials and methods.

at low ligand concentrations, as measured using ERE-regulated reporters (Hall & McDonnell 1999). A conclusion from these data is that ERβ can directly modulate ERα activity. This has significance since many reports exist of differential expression of the two receptors under conditions of altered estrogen sensitivity. For example, ERβ expression is significantly downregulated and ERα expression upregulated during human breast tumorigenesis, suggesting that ERβ's ability to modulate ERα is significantly altered during breast tumorigenesis (Leygue *et al.* 1998*b*, Roger *et al.* 2001). In addition, current data show that in normal and neoplastic breast tissues, the level of expression of the C-terminally truncated ERβ variants, ERβ2 and ERβ5, is markedly higher than the ligand binding ERβ1. These data suggest that the variant ERβ isoforms may also have a role in modulating estrogen and possibly antiestrogen action in human breast cells. The experiments described in this manuscript were undertaken to gain insight into the possible role of the truncated ERβ variants.

Our data show that only hERβ1 is able to bind ligand. Steroid hormone receptors are known to undergo conformational changes during the process of activation especially due to ligand binding, and differences are seen between agonist and antagonist binding (Beekman *et al.* 1993, McDonnell *et al.* 1995). Recent structural analyses of the ligand binding domain (LBD) of several nuclear receptors suggest that the LBD contains common structural motifs that generate a conserved ligand binding pocket, and that agonists and antagonists bind to the same site but induce different conformational changes that are now known to affect transcriptional function, providing structural evidence for antagonism (Brzozowski *et al.* 1997). The variant hERβ isoforms, while not binding ligand, may exist in an activated state in the absence and presence of ligand; however, our data suggest that hERβ2 and hERβ5 are unlikely to be in an activated conformation, and this is consistent with their inability to activate transcription of either a 'classical' or a 'non-classical' estrogen receptor regulated reporter gene.

All ERβ isoforms examined (ERβ1, ERβ2, and ERβ5) inhibit the transcriptional activity of ERα on ERE-containing promoters, while only ERβ1 has any activity alone. This confirms and extends previous data and demonstrates that the relative inhibitory activity of the ERβ isoforms is

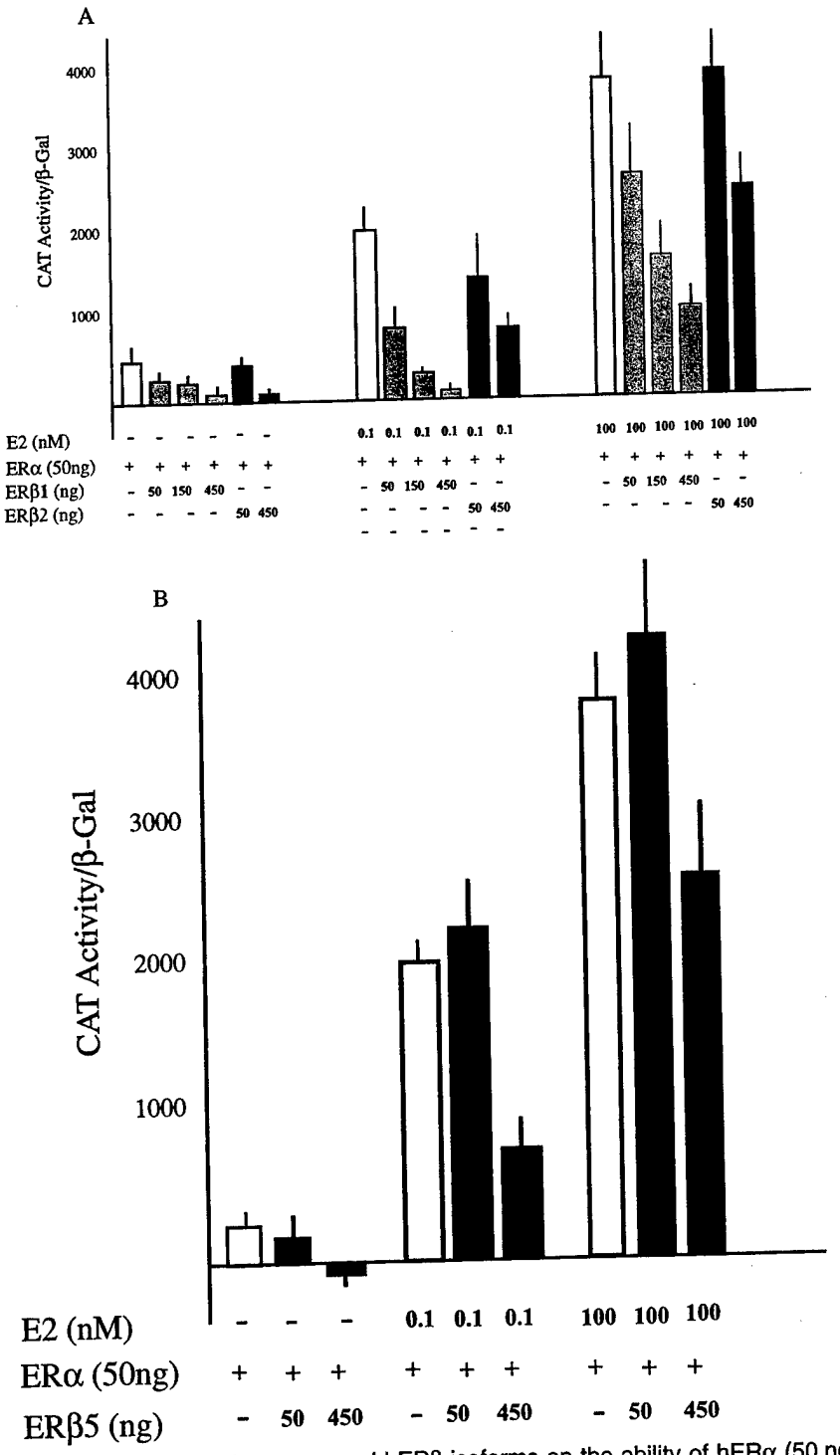


Figure 7 Effect of increasing amounts of coexpressed hERβ isoforms on the ability of hERα (50 ng) to activate transcription from an ERE (vitellogenin A2) regulated CAT reporter gene in the presence and absence of ligand following co-transfection into Cos-1 cells. The results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency)±S.E.M. of 3 independent experiments. (A) Effect of hERβ1 (ERβ1) and hERβ2 (ERβ2) on hERα (ERα). (B) Effect of hERβ5 (ERβ5) on hERα. See text for statistical analysis.

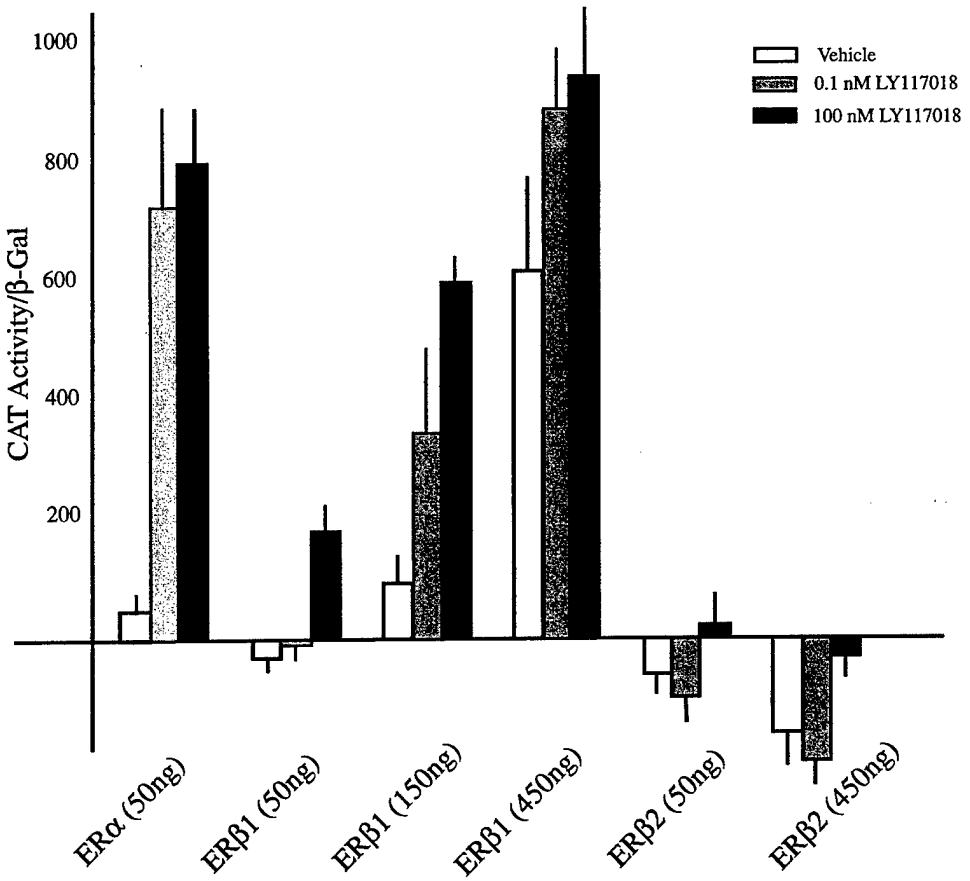
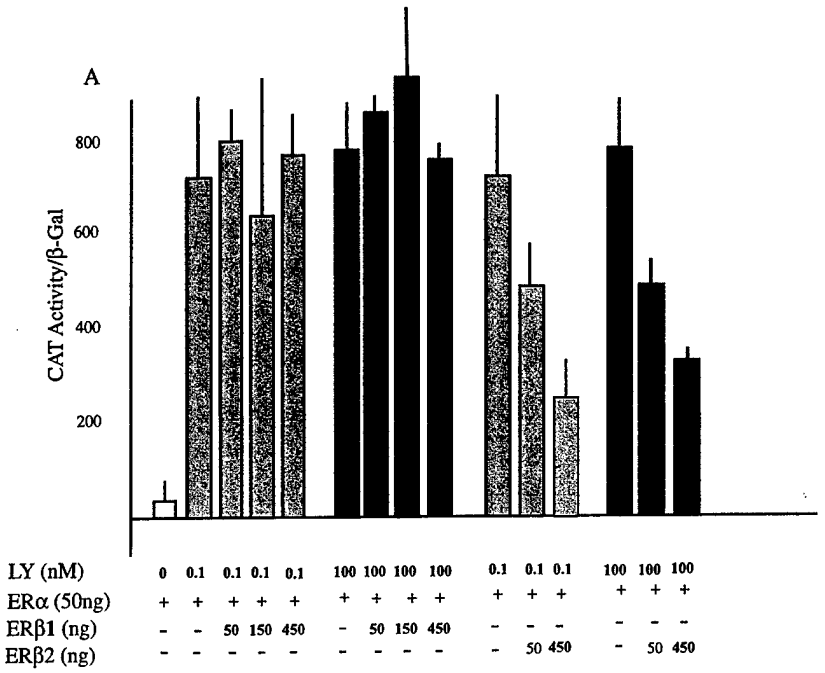
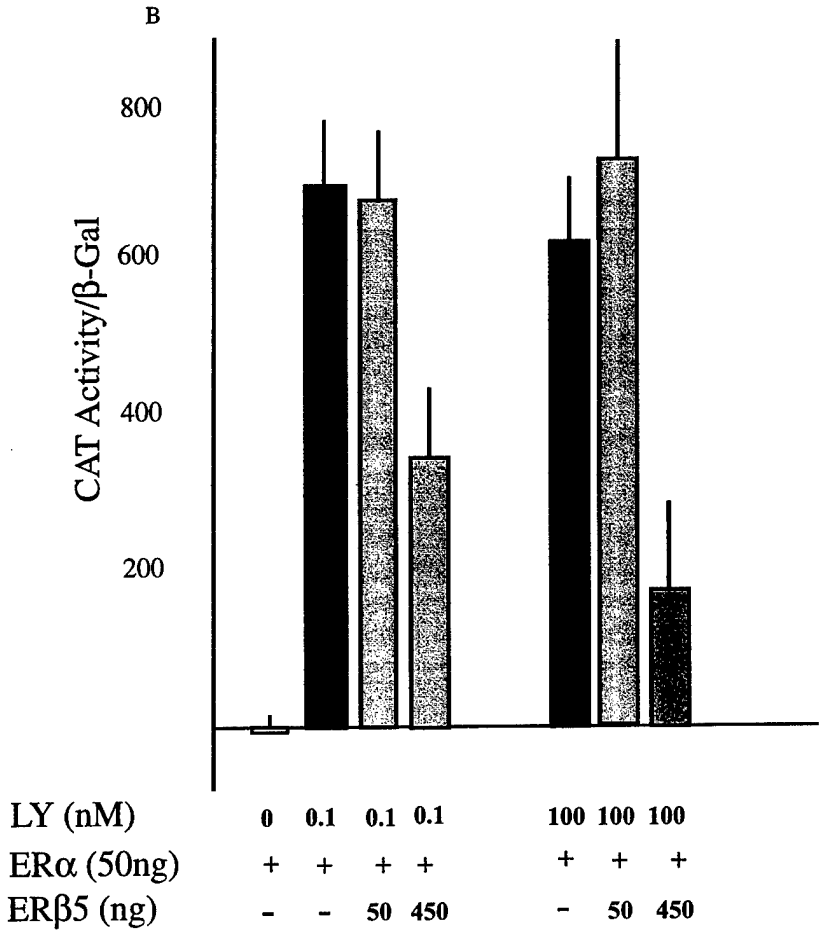


Figure 8 Transcriptional activity of hERα (ERα), hERβ1 (ERβ1) and hERβ2 (ERβ2) on a novel raloxifene responsive element regulated reporter gene, TGF-β3-CAT. Increasing amounts of hER isoform expression vectors were co-transfected with a TGF-β3-CAT reporter gene into Cos-1 cells. The transfected cells were treated for 48 h with or without the raloxifene analog LY117018. The results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency)±S.E.M. of 3 independent experiments. See text for statistical analysis.

ERβ1>ERβ2>ERβ5. This correlates with the relative efficiencies with which ERβ homodimers bind to DNA and may suggest a competition of the beta isoform homodimers with ERα homodimers for DNA binding. However, since heterodimers are preferred under these conditions, it is likely that these predominate under our experimental conditions and the intrinsically lower transcriptional activity of the heterodimers are predominant. Cowley *et al.* (1997) demonstrated that when hERα and hERβ1 are expressed at both a 1:1 and 1:2 ratio the ERα/ERβ1 heterodimer was predominant. This heterodimer had a DNA binding affinity similar to that of the ERα homodimer, and was capable of recruiting steroid receptor coactivator-1 (SRC-1). However, the heterodimer has less transcriptional activity than the ERα/ERα homodimer, suggesting that it may be less efficient in recruiting coactivators than the ERα homodimer. In contrast, the C-terminally truncated hERβ2 has markedly reduced ability to bind to DNA and likely the ERα/ERβ2 heterodimer also binds less well than ERα/ERα homodimers to an ERE (Moore *et al.* 1998, Ogawa *et al.* 1998*b*). But in contrast to hERβ1, hERβ2 does not recruit coactivators (Ogawa *et al.* 1998*b*). Our data show that hERβ5 is less efficient than hERβ2 in binding to DNA, and is also unlikely to recruit coactivators. However, at an ERE the wild-type hERβ1 is more potent than either of the two variants in inhibiting the ability of ERα to activate transcription. So it appears that the inability to


A
CAT Activity/β-Gal
800
600
400
200
LY (nM) 0 0.1 0.1 0.1 0.1 100 100 100 100 0.1 0.1 0.1 100 100 100
ERα (50ng) + + + + + + + + + + + + + + +
ERβ1 (ng) - - 50 150 450 - 50 150 450 - - - - - -
ERβ2 (ng) - - - - - - - - - - 50 450 - 50 450


B
CAT Activity/β-Gal
800
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LY (nM) 0 0.1 0.1 0.1 100 100 100
ERα (50ng) + + + + + + +
ERβ5 (ng) - - 50 450 - 50 450

recruit coactivators is not correlated with the ability of ERβ isoforms to inhibit ERα activity. Since DNA activity is also a reflection of efficiency of dimerization, it is speculated that the truncated ERβ isoforms have reduced ability to dimerize with ERα and form stable heterodimers than the wild-type ERβ1. Together with our Western blot data it seems that significant inhibition of ERα transcription occurs at levels of ERβ1 expression that are less than or equivalent to ERα (50 ng ERβ1 plasmid makes less protein than 50 ng ERα plasmid, but still significantly affects ERα transcription activity). Therefore our data would be consistent with the mechanism of inhibition being related to a high efficiency of dimerization and reduced efficiency in recruiting coactivators, but not the inability to recruit coactivators.

Interestingly, marked differences in the ability of the ERβ isoforms to affect ERα activity are seen at an estrogen receptor responsive site where the mechanism of transcriptional regulation is quite distinct from that operating at a classical ERE, e.g. the so-called raloxifene responsive element in the TGF-β3 promoter (Yang *et al.* 1996). This is in marked contrast to the results seen at an ERE regulated reporter gene. The ER responsive site in the TGF-β3 promoter is poorly activated by the estradiol–ERα complex, but is strongly activated by the raloxifene–ERα complex. In addition, the DNA binding domain of the ER is not required for this activation. It is assumed that protein–protein interactions between ERα and other transcription factors bound to this promoter are involved in regulation. However, the identity of these 'other' transcription factors is unknown. Using an analog of raloxifene, LY117018 (Lu *et al.* 2000), we have confirmed that this promoter is poorly activated by the estradiol–ERα complex (and this was not altered in our hands by treatment of the transfected Cos-1 cells with epidermal growth factor (Lu & Giguere 2001); data not shown) but was significantly activated by the LY117018–ERα complex. Similarly, the LY117018–hERβ1 complex was found to activate transcription from the TGF-β3 promoter, but in contrast to the murine ERβ1 (Lu *et al.* 2000), is less active than the LY117018–hERα complex. Human ERβ2 and hERβ5 alone could not activate this promoter. This is in contrast to the murine ERβ2 variant (Lu *et al.* 2000) which is structurally quite different to the hERβ2. Furthermore, no murine equivalent to either hERβ2 or hERβ5 isoforms, that are frequently expressed in human tissues, has as yet been identified. However, coexpression of increasing amounts of hERβ2 and hERβ5 with ERα resulted in inhibition of LY117018–ERα transcriptional activity but not LY117018–ERβ1 activity from the TGF-β3 promoter. In contrast to an ERE-containing promoter is the observation that the wild-type hERβ1 did not significantly inhibit the transcriptional activity of the LY117018–ERα complex at the TGF-β3 promoter. At this promoter the differences in the hERβ isoform activity on LY117018–ERα complexes were correlated to the ability to recruit coregulatory factors. Significant effects of hERβ2 on hERα were seen under conditions of equimolar expression, as determined by Western blot analysis of the similarly tagged proteins, but hERβ5 was less active than hERβ2 and this is consistent with a reduced efficiency of dimerization. There appears to be differential expression of hERβ isoforms at least at the RNA level in different human tissues, as well as altered relative expression during breast tumorigenesis (Leygue *et al.* 1999, Omoto *et al.* 2002), and altered levels of hERβcx (hERβ2) as well as hERβ1 during prostate cancer progression (Fujimura *et al.* 2001). Therefore, it is possible that the differential activities of hERβ isoforms on some genes may have both physiological and pathophysiological importance.

In conclusion we have characterized some potential functions of several commonly expressed hERβ isoforms. Generally, the ligand binding wild-type hERβ1 has transcriptional activity alone on both 'classical' and 'non-classical' estrogen responsive promoters, although it is less efficient than ERα. Furthermore, the hERβ family of receptors generally negatively modulate ERα

Figure 9 Effect of increasing amounts of coexpressed hERβ isoforms on the ability of hERα (50 ng) to activate transcription from a TGF-β3-CAT reporter gene in the presence and absence of the raloxifene analog LY117018 following co-transfection into Cos-1 cells. The results show the mean CAT activity after correction for β-galactosidase activity (transfection efficiency)±S.E.M. of 3 independent experiments. (A) Effect of hERβ1 (ERβ1) and hERβ2 (ERβ2) on hERα (ERα). (B) Effect of hERβ5 (ERβ5) on hERα. See text for statistical analysis.

transcriptional activity when coexpressed at 'classical' as well as 'non-classical' ER responsive promoters. However, promoter specific differential activity of the various hERβ isoforms was found, in particular between the wild-type hERβ1 and its C-terminally truncated variants hERβ2 and hERβ5. The possibility that there is differential expression of the hERβ isoforms suggests that they may have a role in differentially modulating estrogen action.

Acknowledgements

This research is supported by the Canadian Breast Cancer Research Initiative. E L holds a career development award (BC000577) from the USAMRMC.

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Received in final form 24 October 2002
Accepted 30 October 2002

Appendix 8 **Brit J Cancer. 2002. 87: 1411-1416**

British Journal of Cancer (2002) 87, 1411–1416

www.bjcancer.com

Relationship of coregulator and oestrogen receptor isoform expression to *de novo* tamoxifen resistance in human breast cancer

LC Murphy*[,1,2], E Leygue[2], Y Niu[3], L Snell[3], S-M Ho[4] and PH Watson[3]

[1]Manitoba Institute of Cell Biology, University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, Canada, R3E OW3; [2]Department of Biochemistry and Medical Genetics, University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, Canada, R3E OW3; [3]Department of Pathology, University of Manitoba, Faculty of Medicine, Winnipeg, Manitoba, Canada, R3E OW3; [4]Department of Surgery, University of Massachusetts Medical School, Worchester. Massachusetts, MA 01655, USA

This study addresses the hypothesis that altered expression of oestrogen receptor-beta and/or altered relative expression of coactivators and corepressors of oestrogen receptors are associated with and may be mechanisms of *de novo* tamoxifen resistance in oestrogen receptor positive breast cancer. All cases were oestrogen receptor +, node negative, primary breast tumours from patients who later had no disease progression (tamoxifen sensitive) or whose disease progressed while on tamoxifen (tamoxifen resistant). Using an antibody to oestrogen receptor-beta that detects multiple forms of this protein (total) but not an antibody that detects only full-length oestrogen receptor-beta 1, it was found that high total oestrogen receptor beta protein expressors were more frequently observed in tamoxifen sensitive tumours than resistant tumours (Fisher's exact test, P=0.046). However, no significant differences in the relative expression of oestrogen receptor β2, oestrogen receptor β5 and full-length oestrogen receptor β1 RNA in the tamoxifen sensitive and resistant groups were found. Also, when the relative expression of two known coactivators, steroid receptor RNA activator and amplified in breast cancer 1 RNA to the known corepressor, repressor of oestrogen receptor activity RNA, was examined, no significant differences between the tamoxifen sensitive and resistant groups were found. Altogether, there is little evidence for altered coregulators expression in breast tumours that are *de novo* tamoxifen resistant. However, our data provide preliminary evidence that the expression of oestrogen receptor β protein isoforms may differ in primary tumours of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy.
British Journal of Cancer (2002) **87,** 1411–1416. doi:10.1038/sj.bjc.6600654 www.bjcancer.com

Keywords: SRA; AIB1; ROA; coregulators; OR isoforms; human breast cancer; tamoxifen

The ability of anti-oestrogens such as tamoxifen to compete with oestrogens for binding to OR and to antagonise their mitogenic action provides the basic rationale for endocrine therapy and prevention (for a review see (Osborne, 1998b) in breast cancer. Adjuvant tamoxifen post-operative therapy reduces the number of recurrences and prolongs survival in women whose primary tumours are oestrogen receptor (OR) positive (Group, 1998). However, even though OR level is considered a marker for predicting the likelihood of responding to adjuvant hormonal therapies, some patients, whose primary tumours are OR positive do not respond to tamoxifen treatment. Such apparent *de novo* tamoxifen resistance does not depend upon the level of OR within the primary tumour. As well many of those patients whose disease initially responds to tamoxifen, progress while still under treatment having acquired resistance and this occurs despite continued expression of OR. Thus suggesting other components of the oestrogen signalling pathway may be altered. Recent observations using laboratory models (Hall and McDonnell, 1999; Lanz *et al*, 1999; McKenna *et al*, 1999; Montano *et al*, 1999) have demonstrated that altered levels of OR isoforms and/or alteration of expression of coactivators and corepressors can deregulate oestrogen and antioestrogen activity in target cells, suggesting the hypothesis that altered levels of OR isoforms and/or coregulators *in vivo* could be a mechanism of tamoxifen resistance. Previously we have demonstrated that the relative expression of ORα/ORβ as well as the relative expression of some OR coactivators to corepressors is significantly altered during breast tumourigenesis *in vivo* (Leygue *et al*, 1998; Murphy *et al*, 2000). Furthermore, since these alterations parallel the marked changes in oestrogen action that accompany breast tumourigenesis, they may have a role in this process. To explore the hypothesis that such changes could underlie *de novo* tamoxifen resistance *in vivo*, the expression of OR isoforms, two known coactivators (steroid receptor RNA activator (SRA), (Lanz *et al*, 1999) and amplified in breast cancer-1 (AIB1) (Anzick *et al*, 1997)) and one corepressor (repressor of oestrogen receptor activity, repressor of oestrogen receptor activity (ROA) (Montano *et al*, 1999)) of OR activity have been investigated in primary breast tumours from node negative patients whose tumours were OR

*Correspondence: Dr LC Murphy; Manitoba Institute of Cell Biology, CancerCare Manitoba, 675 McDermot Ave, Winnipeg, MB. R3E OV9, Canada; E-mail: lcmurph@cc.umanitoba.ca
Received 1 July 2002; revised 4 September 2002; accepted 19 September 2002

positive and that subsequently responded or had disease progression while on adjuvant tamoxifen therapy.

MATERIALS AND METHODS

Human breast tumours

All breast tumour cases used for this study were selected from the NCIC-Manitoba Breast Tumour Bank (Winnipeg, Manitoba, Canada). As previously described (Hiller *et al*, 1996), tissues are accrued to the Bank from cases at multiple centres within Manitoba, rapidly collected and processed to create matched formalin-fixed-embedded and frozen tissue blocks for each case with the mirror image surfaces oriented by coloured inks. The histology of every sample in the Bank is uniformly interpreted by a pathologist in Hematoxylin/Eosin (H&E) stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific paraffin and frozen blocks from cases for this study. For each case, interpretations included an estimate of the cellular composition (including the percentage of invasive epithelial tumour cells and stroma), tumour type and tumour grade (Nottingham score). Steroid receptor status was determined for all cases by ligand binding assay performed on an adjacent portion of tumour tissue. Tumours with oestrogen receptor levels above 3 fmol mg^{-1} of total protein were considered OR positive.

To identify cases that responded divergently to tamoxifen, review of approximately 1000 consecutive cases was undertaken to identify cases that were OR positive, node negative and that had been treated with adjuvant tamoxifen following surgery +/− local radiation. From these the first cohort of 27 cases was selected to include a subset (n=13) that had shown progression of disease (either died or alive with recurrent disease, referred to as tamoxifen resistant cases) and a similar control subset (n=14) specifically selected to comprise cases with similar lengths of follow-up, OR status, tumour grade and tumour histology, but that had shown no progression of disease (referred to as tamoxifen sensitive cases). The tumour characteristics were: (1) 'Tam Sensitive' group median OR was 60.5 fmol mg^{-1} protein (range 6–146 fmol mg^{-1} protein), median PR was 32 fmol mg^{-1} protein (range 8–216 fmol mg^{-1} protein); median grade was five (range 4–8); median age at biopsy was 69 years (range 35–87 years); median follow-up time was 56 months (range 18–79); (2) 'Tam Resistant' group median OR was 57 fmol mg^{-1} protein (range 4–136 fmol mg^{-1} protein); median PR was 14 fmol mg^{-1} protein (range 4–288 fmol mg^{-1} protein); median grade was six (range 4–9); median age at biopsy was 67 years (range 49–83 years); median follow-up time was 56 months (range 9–85).

For the RNA studies, frozen tissue corresponding to the blocks for several of the first cohort of older cases used above, were not available. Therefore, after further review of the tumour bank as described above, a second study cohort was selected that also had frozen tissue available. The relevant patient/tumour characteristics were similar to the above cohort, although the follow-up time was shorter: (1) 'Tam Sensitive' group (n=16) median OR was 37.5 fmol mg^{-1} protein (range 4.4–146 fmol mg^{-1} protein), median PR was 44 fmol mg^{-1} protein (range 13.1–216 fmol mg^{-1} protein); median grade was six (range 4–9); median age at biopsy was 72 years (range 47–87 years); median follow-up time was 39 months (range 13–76); (2) 'Tam Resistant' group (n=16) median OR was 21.5 fmol mg^{-1} protein (range 5.6–107 fmol mg^{-1} protein); median PR was 14.3 fmol mg^{-1} protein (range 7.8–288 fmol mg^{-1} protein); median grade was six (range 4–9); median age at biopsy was 71 years (range 60–89 years); median follow-up time was 34 months (range 9–63).

Immunohistochemistry

Immunohistochemistry was performed on serial 5 μm sections from a representative, formalin fixed paraffin embedded archival tissue block from each tumour. Immunohistochemical staining for ORβ was performed using two different primary antibodies. IgYERB503 (a gift from Dr Jan-Ake Gustafson) detects total ORβ isoforms (Horvath *et al*, 2001; Saji *et al*, 2000) and GC17 (a gift from Dr Shuk-Mei Ho) detects only the full-length ORβ (Leav *et al*, 2001). The GC17 polyclonal antibody was raised in rabbits against a peptide sequence in the F domain of the human OR-β receptor (amino acids 449 to 465) and its specificity validated previously (Leav *et al*, 2001). The epitope to which the IgYERB503 antibody is directed is not known, but this polyclonal chicken antibody was raised to an ORβ recombinant protein which was disrupted in the ligand binding domain by insertion of 18 additional amino acids, but was subsequently shown to also recognise the full-length non-inserted ORβ protein (Saji *et al*, 2000). Antibodies were applied using an automated tissue immunostainer (Discovery module, Ventana Medical Systems, Phoenix, AZ, USA), DAB immunohistochemistry kit and bulk reagents that were supplied by the manufacturer. Briefly, the Discovery staining protocol was set to 'Standard Cell Conditioning' procedure, followed by 12 h incubation with primary antibody and 32 min incubation with secondary antibody. Concentrations of primary antibodies initially applied to the Ventana instrument were 1:200 for IgYERB503 and 1:50 for GC17, which translates into final concentrations of 1:600 and 1:150 after a 1:3 dilution with buffer dispensed onto the slide with the primary antibody. Levels of nuclear ORβ expression were scored semi-quantitatively, under the light microscope. Scores were obtained by estimating average signal intensity (on a scale of 0–300) and the proportion of epithelial cells showing a positive signal (0, none; 0.1, less than one tenth; 0.5, less than one half; 1.0 greater than one half). The intensity and proportion scores were then multiplied to give an overall IHC-score. Cases with a score lower than or equal to 100 were considered negative or weakly positive, whereas tumours with scores higher than 100 were classified as positive for ORβ expression (Al-Haddad *et al*, 1999).

RNA Extraction and RT–PCR conditions

Total RNA was extracted from 20 μm frozen tissue sections (20 sections per tumour) using Trizol™ reagent (Life Technologies, NY, USA) according to the manufacturer's instructions and quantified spectrophotometrically. One μg of total RNA was reverse transcribed in a final volume of 25 μl as previously described (Leygue *et al*, 1996).

Primers and PCR conditions

Coregulators The primers used were: SRAcoreU primer (5′-AGGAACGCGGCTGGAACGA-3′; sense; positions 35–53, Genbank accession number AF092038) and SRAcoreL primer (5′-AGTCTGGGGAACCGAGGAT-3′; antisense; position 696–678, Genbank accession number AF092038); AIB1-U primer (5′-ATACTTGCTGGATGGTGGACT-3′; sense; positions 110–130, Genbank accession number AF012108) and AIB1-L primer (5′-TCCTTGCTCTTTTATTTG ACG-3′; antisense; positions 458–438, Genbank accession number AF012108); ROA-U primer (5′-CGAAAAATCTCCTCCCCTACA-3′; sense; positions 385–405, Genbank accession number AF150962) and ROA-L primer (5′-CCTGCTTTGCTTTTTCTACCA-3′; antisense; positions 781–761, Genbank accession number AF150962).

Radioactive PCR amplifications for SRA were performed and PCR products analysed as previously described (Leygue *et al*, 1999b) with minor modifications. Briefly, 1 μl of reverse transcrip-

tion mixture was amplified in a final volume of 15 μl, in the presence of 1.5 μCi of (α-^{32}P) dCTP (3000 Ci mmol^{-1}), 4 ng μl^{-1} of each primer and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA). For SRA each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and exposed 2 h to a Molecular Imager™-FX Imaging screen (Bio-Rad, Hercules, CA, USA).

PCR amplifications for AIB1 and ROA were performed and PCR products analysed as previously described (Leygue *et al*, 1996) with minor modifications. Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 20 μl, in the presence of 4 ng μl^{-1} of each primer and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA). For AIB1, each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 55°C and 30 s at 72°C). For ROA each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 57°C and 30 s at 72°C). PCR products were then separated on agarose gels stained with ethidium bromide as previously described (Leygue *et al*, 1996).

Primers for OR isoforms

ORα-U primer (5′-TGTGCAATGACTATGCTTCA-3′; sense; located in ORα 792–811) and ORα-L primer (5′-GCTCTT-CCTCCTGTTTTTA-3′; antisense; located in ORα 940–922). Nucleotide positions given correspond to published sequences of the human ORα cDNA (Green *et al*, 1986). PCR amplifications were performed and PCR products analysed as previously described with minor modifications (Dotzlaw *et al*, 1997). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl, in the presence of 1 μCi (α-^{32}P) dCTP (3000 Ci mmol^{-1}), 2 ng μl^{-1} of ORα-U/ORα-L and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA). Each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C and 30 s at 72°C).

A previously validated triple primer assay was used to determine the relative expression of ORβ1 and its variant isoforms ORβ2 and ORβ5 (Leygue *et al*, 1999a). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl, in the presence of 1 μCi of (α-^{32}P) dCTP (3000 Ci mmol^{-1}), 4 ng μl^{-1} of each primer (ORβ1U, ORβ1L and ORβ2L) and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA).

All OR PCRs consisted of 30 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7 M urea. Following electrophoresis, the gels were dried and autoradiographed. Three independent PCRs were performed.

Quantification of SRA and OR RNA expression Exposed screens were scanned using a Molecular Imager™-FX (Bio-Rad, Hercules, CA, USA) and the intensity of the signal corresponding to SRA or the appropriate OR isoform PCR fragments was measured using Quantity One™ software (Bio-Rad, Hercules, CA, USA). Three independent PCRs were performed. In order to control for variations between experiments, a value of 1 was arbitrarily assigned to the signal of one particular tumour measured in each set of PCR experiments (always the same tumour) and all signals were expressed relative to this signal. Levels of SRA was expressed relative to ROA (SRA/ROA), AIB1 (SRA/AIB1) or ORα (SRA/ORα) in each individual tumour sample. Levels of ORβ isoforms were expressed relative to other ORβ isoforms shown under statistical analysis and as previously described (Leygue *et al*, 1999a).

Quantification of the relative expression of the deleted SRA variant RNA It has previously been shown that the coamplification of a full-length and a deleted variant SRA (SRA-Del) cDNA resulted in the amplification of two PCR products, the relative signal intensity of which provided a reliable measurement of the relative expression of the deleted variant (Leygue *et al*, 1999b). For each sample, the signal corresponding to the SRA-Del was measured using Quantity One™ software (Bio-Rad, Hercules, CA, USA) and expressed as a percentage of the corresponding core SRA signal. For each case, three independent assays were performed and the mean determined.

Quantification of ROA and AIB1 RNA expression Following analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst™ (Bio-Rad, Hercules, CA, USA). At least, three independent PCRs were performed. A value of 1 was arbitrarily assigned to the ROA or AIB1 signal of one particular tumour and is the same tumour as described above and all signals were expressed relative to this signal. Levels of AIB1 were expressed relative to ROA (AIB1/ROA) and ORα (AIB1/ORα), and levels of ROA were expressed relative to ORα (ROA/ORα).

Statistical analysis Differences between tamoxifen sensitive and tamoxifen resistant cases were tested using the Mann–Whitney rank sum test, two-tailed. Potential differences in expression between the two groups with respect to each ORβ isoform RNA relative to other ORβ isoform RNA expression (e.g. log ORβ1/total ORβ; log ORβ2/total ORβ; log ORβ5/total ORβ, as previously described (Leygue *et al*, 1999a)), and the relative expressions of coregulators (i.e. logAIB1/ROA; logSRA/ROA; logSRA/AIB1; logAIB1/ORα; logSRA/ORα; logROA/ORα) were determined.

Tumours were classified as low (scores between 0 and 100) and high (150–300) ORβ expressors, and differences between tamoxifen sensitive and tamoxifen resistant cases were tested using Fisher's exact test. Correlation between ORβ protein expression (IHC-score) and tumour characteristics was tested by calculation of the Spearman coefficient *r*.

RESULTS

Expression of ORβ protein in primary human breast tumours from patients who later progressed on tamoxifen treatment or showed no progression on tamoxifen treatment

ORβ protein was determined immunohistochemically on adjacent sections from each tumour, using two different antibodies. GC-17 is an antibody recognizing an epitope in the C-terminus of full-length ORβ1 (Leav *et al*, 2001). Normal breast tissue was used as a positive control and is shown in Figure 1A. Examples of staining in human breast tumour sections are shown in Figure 1B–D. Some tumour sections showed no (Figure 1B, full-length ORβ score=0) or low (Figure 1C, full-length ORβ score=100), while others showed strong full-length ORβ signals (Figure 1D, wild-type ORβ score=300). Tumours were classified as low (scores between 0 and 100) and high (150–300) full-length ORβ protein expressors, and differences between tamoxifen sensitive and resistant tumours determined by Fisher's exact test. No significant differences were found.

IgYERbeta503 is an antibody that recognises ligand binding and non-ligand binding ORβ protein isoforms (Horvath *et al*, 2001; Saji *et al*, 2000) and which we refer to as total ORβ protein. Normal breast tissue was used as a positive control and is shown in Figure 2A. Examples of staining with this antibody in human breast tumour sections are shown in Figure 2B–D. Some sections showed no (Figure 2B, total ORβ score=0) or low (Figure 2C, total ORβ score=100) total ORβ expression whereas others had strong total ORβ protein signal (Figure 2D, total ORβ score=300).

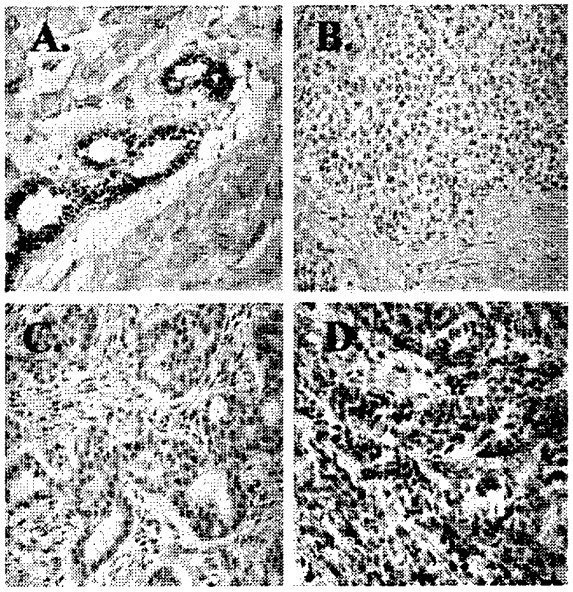

Figure 1 Examples of immunohistochemistry using the GC-17 antibody which only recognises the full-length ORβ1: (**A**) normal human breast tissue; (**B**) ORβ1 negative human breast tumour, H-score=0; (**C**) ORβ1 low expressing human breast tumour, H-score=100; (**D**) ORβ1 high expressing human breast tumour, H-score=150.

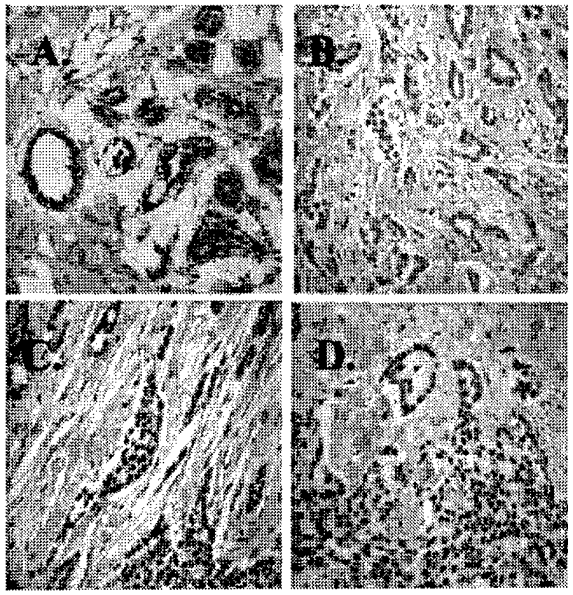

Figure 2 Examples of immunohistochemistry using the IgYERβ503 antibody which recognises most ORβ isoforms: (**A**) normal human breast tissue; (**B**) ORβ negative human breast tumour, H-score=25; (**C**) ORβ low expressing human breast tumour, H-score=100; (**D**) ORβ high expressing human breast tumour, H-score=225.

Tumours were classified as low and high total ORβ protein expressors, and there was a statistically significant difference in high total ORβ protein between the Tamoxifen sensitive and resistant groups (Fisher's exact test, P=0.046). High total ORβ protein expressors were more frequently observed in tamoxifen sensitive tumours than resistant tumours.

Correlation between ORβ protein expression and tumour characteristics was tested by calculation of the Spearman coefficient. A positive correlation between ORβ1 (GC17) protein and progesterone receptor (PR) levels (Spearman r=0.44, P=0.022) was found when each was examined as continuous variables. When tumours were divided into PR+ (>10 fmol mg^{-1} protein) or PR− ($\leqslant 10$ fmol mg^{-1} protein) groups there was a significantly higher level of ORβ1 (GC17) protein in PR+ tumours compared to PR− tumours (Mann–Whitney test, P=0.0268; median for PR+ tumours=55, range 5–150 and median for PR− tumours=10, range 0–75). As well, there was also a significantly higher level of total ORβ (IgY503) protein in PR+ tumours compared to PR− tumours (Mann–Whitney test, P=0.0085; median for PR+ tumours=125, range 25–270 and median for PR− tumours=50 range 0–100).

Relative expression of ORβ isoform RNA in primary human breast tumours from patients who later progressed on tamoxifen treatment or showed no progression on adjuvant tamoxifen To determine if the differences described above in ORβ protein expression were correlated with differences in ORβ variant isoform RNA expression, we compared the relative expression of ORβ variant RNA to full-length ORβ RNA in the tamoxifen sensitive and resistant groups. Unfortunately, frozen tissue samples corresponding to many of the paraffin blocks from patients in the cohort used for immunohistochemistry were not available. Therefore additional cases selected were selected from the tumourbank as described in Materials and Methods. Using previously validated assays (Leygue *et al*, 1998; 1999a) the relative expression of ORβ2, ORβ5 and full-length ORβ1 RNA in the tamoxifen sensitive and resistant groups was not significantly different.

Relative expression of coregulators in primary human breast tumours from patients who later progressed on tamoxifen treatment or showed no progression on tamoxifen treatment To address the hypothesis that altered relative expression of steroid receptor coactivators and corepressors could underlie altered tamoxifen sensitivity in human breast tumours, and since we previously showed that the relative expression of two coactivators (SRA and AIB1) to a corepressor (ROA) is altered in OR+ breast tumours compared their adjacent normal breast tissue, we chose these coregulators to study. They were measured by RT–PCR in the above tumour cohorts. SRA, AIB1, and ROA mRNAs were detectable in most samples, even though their level of expression differed from one sample to another. Consistent with our previous results (Leygue *et al*, 1999b), an additional fragment, migrating at an apparent size of 459 bp was also observed in most tumours when using SRA specific primers. Sequencing analysis revealed that this band corresponded to a variant form of SRA (referred to as SRA-Del) deleted in 203 bp between positions 155 and 357 (positions given correspond to Genbank accession number AF092038). There were no significant differences between the tamoxifen sensitive and the *de novo* tamoxifen resistant breast cancers in the relative expression of any of the coactivators to corepressor RNA, or in the relative expression of SRA/AIB1 RNA, or in expression of any of these coregulator RNAs relative to ORα or total ORβ RNA expression. As well, there was no significant difference in the relative expression of variant SRA/full-length SRA between the groups either.

Tumour characteristics No statistically significant differences were found between the tamoxifen sensitive and tamoxifen resistant cohorts in any of the tumour characteristics described in the Materials and Methods section except for PR. PR levels were statistically significantly different (P=0.044) between the two groups

using a Mann–Whitney rank sum test (two sided). PR levels were higher (median PR was 32 fmol mg^{-1} protein; range 8–216 fmol mg^{-1} protein) in the tamoxifen 'sensitive' group compared to the tamoxifen '*de novo* resistant' group (median PR was 14 fmol mg^{-1} protein; range 4–288 fmol mg^{-1} protein). This was a consistent finding in both selected cohorts (that used for immunohistochemistry and that used for the RNA study), and provides strong support for differences in oestrogen signalling pathways in these two groups since PR is a marker of OR signal transduction (Horwitz *et al*, 1975; Osborne, 1998a).

DISCUSSION

We and others have shown that the relative expression of ORα and ORβ is significantly altered during breast tumourigenesis (Leygue *et al*, 1998; Roger *et al*, 2001), and a similar mechanism has been proposed to underlie tamoxifen resistance in breast cancers (Paech *et al*, 1997). The current study shows no significant differences in expression of full-length ORβ (ORβ1) between tamoxifen sensitive and resistant tumours. Interestingly, in this small cohort of tumours when total ORβ expression was examined, there were significantly more high total ORβ expressors in the tamoxifen 'sensitive' compared to the 'resistant' group. The data suggest the possibility that increased and altered ORβ isoform protein expression may have a role in *de novo* tamoxifen resistance, or at least together with other parameters may provide better markers of endocrine sensitivity. The increased expression of ORβ proteins in the tamoxifen sensitive group is also consistent with recently published data where patients with ORβ positive tumours (determined using an antibody to an N-terminal epitope of the ORβ protein, and defined as nuclear staining in >10% of cancer cells) had a significantly better overall survival than patients with ORβ negative tumours while receiving adjuvant tamoxifen therapy (Mann *et al*, 2001). Both these latter data and those presented currently in this manuscript are in contrast to data showing increased ORβ RNA expression in tamoxifen resistant tumours versus tamoxifen sensitive tumours previously published (Speirs *et al*, 1999). Together these studies suggest that the ORβ status and the nature of ORβ isoforms together with ORα status in human breast cancers may be important biomarkers of endocrine sensitivity, and warrants further study, in larger, prospectively gathered cohorts. The association of increased ORβ isoform expression with tamoxifen sensitivity, suggests a possible mechanistic role, and one possible mechanism may be suggested by several publications which have shown that ORβ isoforms have a modulatory effect on ORα, both in normal tissues (Weihua *et al*, 2000) as well as in cell culture models (Ogawa *et al*, 1998; Hall and McDonnell, 1999).

The potential difference between tamoxifen sensitive and resistant groups with respect to ORβ-like proteins, was not correlated with differences in the relative expression of full-length ORβ and two known variants ORβ2 and ORβ5 at the RNA level between the tamoxifen 'sensitive' versus the tamoxifen 'resistant' groups, however. This may be due to differential regulation of protein versus RNA level or the likelihood that there are other potential ORβ isoforms (known and unknown) expressed in breast tissues in addition to ORβ1, ORβ2 and ORβ5 (Lu *et al*, 1998; Fuqua *et al*, 1999), whose cognate proteins would be detected by the antibody but not measured in the triple primer RT–PCR assay.

Another mechanism for differential tamoxifen sensitivity in OR+ breast tumours could be altered coregulator expression. Although the relative expression of OR coregulators SRA, AIB1 and ROA is altered between normal breast and OR+ breast tumours, there were no significant differences in the ratios of any of the coactivators/corepressors or any of the ratios of these coregulators to ORα RNA levels between primary breast tumours from patients who were later found to be disease free (sensitive) or have disease progression (resistant) while on adjuvant tamoxifen treatment. These data suggest that altered relative expression of these coregulators is unlikely to be a marker of tamoxifen sensitivity in OR+, node negative, primary breast tumours, and unlikely to have a functional role in *de novo* tamoxifen resistance. Although SRA is functional as an RNA molecule, ROA and AIB1 are functional as proteins. Furthermore, other factors can affect protein activity for example phosphorylation in the case of AIB1 (Mora and Brown, 2000) or sequestration by other proteins such as prothymosin-alpha in the case of ROA (Martini *et al*, 2000). Our studies do not exclude differences at the protein and/or activity levels of ROA and AIB1 being involved in *de novo* tamoxifen resistance, nor do they exclude altered expression of these factors having a role in acquired tamoxifen resistance (Lavinsky *et al*, 1998). Altogether, there is little evidence for altered coregulators expression in breast tumours that are *de novo* tamoxifen resistant. However, our data provide preliminary evidence that the expression of ORβ protein isoforms may differ in primary tumours of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy. As well our data support distinct differences in the OR signalling pathways between these two groups of patients since the expression of a known oestrogen responsive gene PR is significantly different between the two groups, the precise mechanisms underlying these differences remain to be elucidated.

ACKNOWLEDGEMENTS

This work was supported by grants from the Canadian Breast Cancer Research Initiative (CBCRI), the Canadian Institutes for Health Research (CIHR) and the US Army Medical Research and Materiel Command (USAMRMC). PH Watson is a recipient of a CIHR Scientist Award and a US Army Academic Award. E Leygue has a USAMRMC Career Development Award.

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Appendix 9 Article, submitted

Inducible Upregulation of Estrogen Receptor-β1 Affects Estrogen and Tamoxifen Responsiveness in MCF-7 Human Breast Cancer Cells.

Leigh C. Murphy*, Baocheng Peng, Anthony Lewis, James R. Davie, Etienne Leygue, Angela Kemp, Kanyarat Ung, Marcello Vendetti, Robert Shiu.

Dept of Biochemistry & Medical Genetics (LCM, AL, BP, JD, EL), Manitoba Institute of Cell Biology (LCM, AL, BP, JD, RS, EL, AK, KU), and Dept of Physiology (MV, RS), University of Manitoba, Winnipeg, Canada.

Short title: Over-expression of Estrogen Receptor-β1 in MCF-7 cells.

* To whom correspondence should be sent:
Dr Leigh Murphy
Manitoba Institute of Cell Biology
University of Manitoba
675 McDermot Ave
Winnipeg. MB
Canada, R3E 0V9.

Telephone: 204-787-4071
Fax: 204-787-2190
E-mail: lcmurph@cc.umanitoba.ca

Keywords: estrogen receptor-β; progesterone receptor; tamoxifen sensitivity;breast cancer

Grant Support: This work was support by the Canadian Breast Cancer Research Initiative (CBCRA) and the USAMRMC. AL holds a CCMF/Guardian Angel Studentship Award.

ABSTRACT.

To investigate the effect of altered ERα and ERβ expression on estrogen and antiestrogen action in breast cancer, we have stably expressed an inducible (Tet-on) ERβ1 in MCF-7 breast cancer cells. Stably expressing clones were isolated and overexpression of ERβ1 correlated with increased levels of specific radiolabeled E2 binding. Increased ERβ1 did not affect endogenous levels of ERα but increased PR levels. Overexpression of ERβ1 reduced growth responses to E2 in contrast to little if any effect of overexpression of ERα. In estrogen replete conditions overexpression of ERβ1 but not ERα reduced proliferation. Overexpression of ERβ1 did not result in antiestrogen resistance but was associated with increased sensitivity to 4-hydroxytamoxifen. Our results suggest that overexpression of ERβ1 in the presence of an endogenously expressed ERα is associated with tamoxifen sensitivity but may negatively modulate ERα mediated growth. However, not all ERα activities are inhibited since endogenous PR expression is increased by both ERα and ERβ1 overexpression. These data parallel those seen in some *in vivo* studies showing a relationship between PR and ERβ expression as well as ERβ expression and tamoxifen sensitivity of ER+ breast cancer patients. These models are relevant and will be useful for dissecting the role of ERβ1 expression in ER+ breast cancer.

INTRODUCTION.

Estrogens are considered to be major driving forces in breast tumorigenesis and breast cancer progression (1, 2). Current evidence suggests that estrogen action is primarily mediated through two receptors, ERα and ERβ(3). These structurally related receptors are generally considered to be ligand regulated transcription factors which classically modulate target gene transcription by binding to estrogen responsive sequences in target gene promoters (4). There is evidence that ERαand ERβ can be expressed together in some cell types and independently expressed in others(5, 6). Based on studies in which ERβ expression was determined at the protein level by immunohistochemistry (IHC)(7-11), and ERαwas determined by IHC or by enzyme linked immunoassay (EIA), an estimated frequency of ERαand ERβstatus in breast tumors was obtained (12). The most frequently occurring status is ERαpositive/ERβ positive (59%) with similar frequencies of between 11-17% for the other three ER phenotypes. It is important to note that there are two groups of ERβ expressing breast tumors, those with co-expression of ERα, and those that express ERβ alone. The former occurs most frequently, and under experimental conditions when ERα and ERβ are coexpressed, they form heterodimers preferentially over homodimerization(13). It is likely that homodimers of ERα, homodimers of ERβ, and ERα/ERβ heterodimers will differentially affect gene expression. Further, transient coexpression of ERα and ERβ in cell lines results in ERβ induced reduction of ERα activity at low ligand concentrations, as measured using ERE-regulated reporters(14) suggesting that ERβ can directly modulate ERα activity in an inhibitory fashion. This may be a mechanism for differential estrogen sensitivity and some reports have documented the differential expression of these two receptors under conditions of altered estrogen sensitivity(15, 16). Interestingly, ERβ expression is downregulated and ERα expression upregulated during human breast tumorigenesis, suggesting that ERβ's ability to modulate ERα may be altered during breast tumorigenesis(15, 17). This correlates with altered estrogen sensitivity and activity occurring during breast tumorigenesis(18). In addition, altered estrogen action can occur during breast cancer progression and this is thought to underlie the development of

tamoxifen resistance(19). Increased expression and/or activity of ERβ has been suggested as a mechanism of tamoxifen resistance, but the data are controversial (7, 20-22).

To investigate further the effect of altered relative expression of ERα and ERβ on estrogen and antiestrogen action in human breast cancer cells, we have stably expressed a tetracycline-inducible (TET-ON) human ERβ1 in MCF-7 human breast cancer cells, which endogenously express high ERα but low ERβ levels(5, 23). The effect of altering the relative expression of these two ERs on estrogen and antiestrogen regulated proliferation, as well as on endogenous markers of ER activity such as progesterone receptor(24) has been determined in this model system.

MATERIALS AND METHODS.

Plasmids.

Epitope-tagged tetracycline-inducible human ERβ1 (full-length) expression plasmids were generated. Human ERβ1 long form of 530 amino acids(25, 26) was tagged at its N-terminus with a polyhistidine and an Xpress™ epitope tag using the pcDNA4/HisMax(A) plasmid (Invitrogen Canada Inc, Burlington, ON) and characterized as previously described(27). This cDNA was then subcloned downstream of the tetracycline-inducible promoter in the pTRE2hyg plasmid(28) (Clontech Laboratories Inc, Palo Alto, CA). This construct contains the selectable hygromycin resistance gene on the same plasmid as the inducible-transgene.

Hormones and reagents. 17β–estradiol and the tetracycline-analog doxycycline were obtained from Sigma Chemical Co (Oakville, ON). Hygromycin B was obtained from Clontech and G418 was from Invitrogen Canada.

Cell culture and transfection. The cells were routinely cultured in DMEM containing 5% (v/v) fetal calf serum (FBS), 0.4% w/v glucose, glutamine and penicillin-streptomycin and 10 nM E2 (5%CM) as previously described (27). To obtain estrogen depleted cells, the culture medium of stock cells (approximately 50% confluent) was changed to phenol red-free DMEM supplemented with 5% (v/v) twice charcoal dextran-treated FBS, glucose, glutamine and penicillin-streptomycin (5%CS) and replaced every 2 days. The cells were grown for a least a week and not more than two weeks in this estrogen depleted medium, and are passaged once during this time. When estrogen depleted cells are around 50% confluent, they are harvested and used for experiments.

For transfection, MCF 7-clone 89 cells (29) were grown in 10%CM in 100 mm dishes until 90% confluent. The cells were then split into 3 x 100 mm dishes and the next day transfected (2-4 μg linearized plasmid per dish) using the Effectene transfection reagent according to the manufacturer's instruction (QIAGEN, Mississauga, ON). Two days after transfection the medium was changed to 10%CM plus 500 μg/ml G418 and 400 μg/ml hygromycin, and the medium was changed every day for 4 days and then every 3 days. Approx. 2 weeks later hygromycin resistance colonies began to appear and 120 large, healthy colonies were transferred to 96 well plates and screened after suitable numbers of cells were achieved as described below.

Generation of MCF-7 human breast cancer cells with doxycycline-inducible epitope tagged human ERβ1.

The generation and characterization of MCF 7 cells stably expressing rTA (reverse tetracycline transactivator, clone 89rTA) have been previously described(29). These cells (clone 89) were transfected and selected as described above. Resistant clones were screened by Western blotting for doxycycline inducible His-Xpress-ERβ1 expression, using anti-Xpress antibody (1/5000 in TBST, Cat#R910-25, Invitrogen Canada Inc, Burlington, ON) as previously described (27). Multiple positive clones were isolated and two independent clones were selected for further characterization.

RNA Extraction and RT-PCR conditions. Total RNA was extracted from frozen cell pellets using Trizol™ reagent (Life Technologies, NY) according to the manufacturer's instructions. One mg of total RNA was reverse transcribed, as previously described (30).

The primers used consisted of PR-U primer (5'-CACAAAACCTGACACCTCCAGTTC-3'; sense; located in PR 2290-2313) and PR-L primer (5'-GCAAAATACAGCATCTGCCCAC-3'; antisense; located in PR 2490-2511). Nucleotide positions given correspond to sequences of the human PR cDNA (accession number M15716). PCR amplifications were performed and PCR products analyzed as previously described(30). Each PCR consisted of 28 cycles (30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C). PCR products were then resolved by electrophoresis on 1.5% agarose gels. Following electrophoresis, the gels were stained with ethidium bromide, as previously descibed(30). Amplification of the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was performed in parallel and PCR products

visualized on agarose gels stained with ethidium bromide as previously described(30). Three independent PCRs for PR and GAPDH, were performed for each experiment. Signals visualized by UV irradiation on a GelDoc 2000/ChemiDoc System (Bio-Rad) were quantified by densitometry using the Quantity One Software (version 4.2, Bio-Rad). PR was normalized to GAPDH in each experiment as previously described(30).

Whole cell ligand binding assays.

Estrogen receptor ligand binding assays were done using the whole cell binding method as previously described (31) following estrogen depletion of the cells by two passages in 5%CS as previously described (31).

Western blotting.

Western blotting was carried out essentially as previously described(27, 32) with minor modifications. ERα was detected using 1/1000 dilution of an ERα mouse monoclonal antibody (NCL-ER-6F11, Novacastra Labs Ltd, Newcastle on Tyne) in TBST buffer (20 mM Tris base; 0.137 M NaCl, pH 7.5, 0.1% v/v Tween-20), PR was detected using 1/1000 dilution of a PR mouse monoclonal antibody (NCL-PGR-AB, Novacastra Labs Ltd, Newcastle on Tyne) in TBST and alpha-tubulin was detected using 1/12000 dilution of tubulin-alpha Ab2 (mouse monoclonal, clone DM1A, Neomarkers, Fremont, CA). All membranes were incubated for 1 hour at room temperature in 0.2% I-block (Tropix, Bedford, MA) in TBS. The blot was washed for 10 minutes at room temperature in TBST and then incubated with the appropriate dilution of primary antibody overnight at 4°C. This incubation was followed by 5 X 5 minute washes in TBST, and then incubation with secondary antibody for 1 hour at room temperature (goat anti-mouse IgG-HRP, Jackson Immuno Research Labs Inc, PA; 1/5000 in TBST). The blot was washed 5 X 5 minutes with TBST and the signal visualized using Supersignal West Dura Extended Duration Substrate kit (Pierce, IL) according to the manufacturer's instructions. Signal detection and documentation were using the ChemiDoc Gel Documentation System 2000 (Biorad Labs Canada, Mississauga, ON).

Immunofluorescence.

Eleven days prior to slide preparation, stock cells were placed in medium containing 5% tetracycline-free FBS (BD Bioscience Clontech, Palo Alto, CA), and the medium was changed every 3 days. These cells were then harvested and 1 x 10^4 cells in 1 ml of

medium were added to each well of four chambered Falcon^{R} Culture Slides (Becton Dickinson, Franklin Lakes, NJ). For every cell line used one well was treated with vehicle alone and the duplicate well was treated with 1 μg/ml of doxycycline for 48 hours. Each chamber slide consisted of two wells of control MCF7 clone 89 cells, and two wells of a His-Xpress-ERβ1 transgenic clone cells. Following incubation, the medium was removed by aspiration and the slides washed with 1 x phosphate buffered saline (PBS) for 2 minutes. The PBS was removed and the cells were fixed and permeabilized with paraformaldehyde solution (3.7% formaldehyde, 0.18% Triton-X-100 in PBS) for 30 minutes at 37° C. The slides were then washed in 1 x PBS for 5 minutes at room temperature, with agitation. Slides were then removed from the washing solution and while still damp, were coated with a hydrophobic barrier around the outside edge using an ImmEdge^{tm} pen (Vector Laboratories Inc, Burlingame, CA). Inside the barrier the cells were covered in 1 ml of blocking solution (1% w/v BSA, 1% v/v normal goat serum in PBS) and incubated for 30 minutes at 37° C in a humidified chamber to prevent evaporation. The blocking solution was removed and the cells incubated with mouse anti-Xpress antibody (1/750 in blocking solution, Invitrogen) for 1 hour at room temperature. The slides were then washed 3 X with PBS for 5 minutes with agitation. Slides were then moved to a dark room and then incubated with CY3-labelled goat anti-mouse secondary antibody (1/10000 in blocking solution) for 1 hour at room temperature. This was followed by 3 X 5 minute washes at room temperature with PBS with agitation. The cells were stained with Hoechst dye (1/10000 in blocking solution) for 20 minutes at room temperature, followed by two washes in PBS for 6 minutes at room temperature. Slides were allowed to stand in PBS for a further 20 minutes at room temperature, then drained and one drop of Fluorsave^{TM} reagent (Calbiochem, Hornby, ON) per well applied. A coverslip was then added and sealed with clear nail polish. The slides were stored in the dark overnight at 4°C and fluorescent images obtained using a Nikon Eclipse E1000 microscope (Nikon, Mississauga, ON) using ACT-1 software (Nikon, Mississauga, ON). All exposure times were for 1 second.

Cell proliferation assay. Cells depleted of estrogen for at least a week by passage in 5%CS were set-up at 5 x 10^4 cells per well in 6 well plates in 5%CS. The cells were left overnight and then treated with 2 μg/ml doxycycline or vehicle alone for 48 hours. Cells

maintained in the presence or absence of doxycycline then were either treated with E2 (10 nM) or vehicle alone. Triplicate wells were counted electronically (Beckman Coulter Canada Inc, Mississauga, ON) at the times indicated after the start of E2 treatment. Medium was changed every 2 days.

Cells grown in 5%CM were set-up at 10^4 cells per well in 6 well plates in 5%CM, left overnight and then treated with 2 μg/ml doxycycline or vehicle alone for 48 hours. Cells were maintained in the presence or absence of doxycycline and treated with varying concentrations of 4-OH-tamoxifen or vehicle alone. Triplicate wells were counted electronically (Beckman Coulter Canada Inc, Mississauga, ON) at the indicated times after the start of treatment. Medium was changed every 2 days. Doubling times were calculated from the initial (n_i) and final (n_t) cell numbers from the equation: doubling time (DT) = $t\log 2/\log (n_i)/(n_t)$, where t is time in days between n_i and n_t. Proliferation rate = DT (control)/DT (treated) x 100 %.

Statistical Analysis. Analysis of variance followed by t-test analyses was used as appropriate.

RESULTS.

Identification of doxycycline-inducible epitope-tagged ERβ1 expressing MCF7 cell lines. Two clones, a low-expressor (β-low) and a high expressor (β-high) shown to express doxycycline-inducible His-Xpress-tagged ERβ1 by Western blot analysis were chosen for further characterization. Controls were the original previously described MCF7 cell line stably expressing rTA (MCF-7)(29) and these MCF-7 cells transfected with the empty pTRE2hyg plasmid alone (V). As an additional control we have included an MCF-7 cell line stably transfected with a doxycycline-inducible GFP-tagged ERα expression vector (α-high)(33, 34). Preliminary experiments showed that induction of the His-Xpress-tagged ERβ1 was dependent on the dose of doxycycline used, and maximum induction in any one clone was achieved by treating the cells for 48 hours with 1-2 μg/ml doxycycline. A Western blot of the cell lines grown in 5%CS ± doxycycline treatment for 48 hours is shown in Figure 1A. A single band of approx. 62 kDa is detected only in the doxycycline treated His-Xpress-tagged ERβ1 transfected lines but not in control cell lines, MCF-7, V-18 and α-high. The level of His-Xpress-tagged ERβ1 expression in each of the clones is different with β-high > β-low. When these cell lines

were grown on glass slides, fixed and the His-Xpress-tagged ERβ1 visualized using anti-Xpress antibody, significant fluorescence was only found over the nucleus (determined by Hoechst staining) of doxycycline treated His-Xpress-tagged ERβ1 clones, with little, if any, signal detected in doxycycline treated MCF-7 cells. An example of the data obtained is shown in Figure 2. These data are consistent with a general localization of the His-Xpress-tagged ERβ1 product in or associated with the nucleus.

Effect of epitope-tagged ERβ1 expression on ERαexpression and estradiol ligand binding assays. Under conditions where the medium was depleted of estrogen (5%CS as described in Materials and Methods), little if any effect of transgene expression on endogenous ERα expression was seen (Figure 1B), and similar amounts of endogenous ERα were expressed in control and tagged ERβ1 over-expressing cells. In order to determine the functional level of ERβ1 over-expression achieved relative to the endogenous level of ER (primarily due to ERα since only very low levels of ERβ have been identified in these cells (5, 35)) under these conditions, whole cell ligand binding assays were carried out on cells grown in 5%CS with and without treatment for 48 hours with 2 μg/ml doxycycline. The results of several independent experiments are presented in Table 1. A significant increase in specific H^3-17β–estradiol binding compared to the non-expressing controls (MCF-7 and V) as well as the no doxycycline treatment controls was measured in all doxycycline treated ERβ1 (β–high, β–low) and the α-high over-expressing cell lines. In addition the levels of binding induced by doxycycline correlated with the level of transgene induced by doxycycline as determined by Western blot analysis, β–high > β–low (Figure 1A). Doxycycline induction of tagged-ERα in α–high cell line under these same conditions led to an increase in ligand binding similar to that obtained in β–high tagged ERβ1cells. These data are consistent with the increased E2 binding being due to the induced expression of tagged ERβ1 or tagged-ERα after 48 hour doxycycline treatment in 5%CS.

Treatment of cells containing doxycycline induced ERβ1 with estradiol-17β (10 nM) for 24 hours resulted in downregulation of the tagged ERβ1 (Figure 3A) in parallel to the expected downregulation of the endogenous ERα (Figure 3B), as did treatment with ICI 182780 (500 nM). In contrast, the tagged ERβ1 product was stabilized by 24 hours of 4-hydroxytamoxifen (500 nM) treatment (Figure 3A). These data are similar to

previously published data demonstrating differential effects of various Selective Estrogen Receptor Modulators (SERMs) on steady state levels of ERα(Figure 3B)(36). The functional consequences of the various ligands were assessed by PR expression and are consistent with previous reports(37, 38). Signals, obtained from Western blot analyses of time course experiments of tagged-ERβ normalized to the tubulin signals from the same blot were qualified by densitometry using the Quantity One software (version 4.2; Bio-Rad). The half life of tagged-ERβusing the line of best fit on a semi-log plot was estimated when bound to various SERMs. The estimated half-life of tagged ERβ1 in the absence of E2 was 7.75 hours, while in the presence of E2 was 2.25 hours. Treatment with 4-hydroxytamoxifen stabilized the receptor increasing its half-life to approx. 28.5 hours, while treatment with ICI182780 resulted in a decreased tagged ERβ1 half-life compared to vehicle treated controls. The estimated half-life of tagged ERβ1 in the presence of ICI182780 was 4.5 hours.

Effect of epitope-tagged ERβ1 expression on endogenous progesterone receptor expression in MCF 7 human breast cancer cells. To determine if tagged ERβ1 expression affected known estrogen responsive endogenous targets, we investigated the level of expression of a known estrogen responsive gene product, the progesterone receptor (PR). Under estrogen depleted conditions in MCF-7 cells the level of PR expression is markedly reduced and usually not detectable using our Western blot conditions. However, after doxycycline treatment PR levels were significantly increased in all ERβ1 over-expressing clones (Figures 3B and 4). Furthermore, the increase in level of PR expression was correlated to the level of transgene expression in the different clones, β-high > β-low (Figure 4). When the cells were treated with E2 for 24 hours following 48 hours of doxycycline, there was a further induction of PR expression in all tagged ERβ1 expressing clones (Figure 3B for β-low, data not shown for β-high) as well as the expected E2 induced PR expression in the non-transgene expressing MCF-7 control cells (data not shown). Since PR is regarded as a gold-standard downstream marker of ERα activity we determined what effects upregulation of α-high would have on PR expression. As shown in Figure 4, increased ERα (α-high) also resulted in an induction of PR expression found under these conditions. A further increase in PR expression was seen in α-high cells with E2 treatment as well (data not shown).

To determine if this apparently ligand independent increase in PR following doxycycline induced tagged-ERβ1 or tagged-ERα was due to effects at the transcriptional level PR mRNA was measured under the conditions described above. PR mRNA levels (determined by RT-PCR) were increased by126 ± 9% in β-low, 148 ± 7% in β-high compared to 93 ± 5% in MCF-7 (mean ± SEM or range, n = 2-7) in a ligand independent manner due to doxycyline induction of the tagged ERβ1 expression. The increased PR mRNA level in the ERβ expressing cells was significant (ANOVA, P = 0.0014) suggesting that the effect of ERβ over-expression on PR protein level was at least in part due to altered steady state levels of RNA suggesting a transcriptional effect.

These data suggest that over-expression of ERβ1 does not inhibit the activity of the endogenous ERα at least, at the level of known marker gene transcription in breast cancer cell lines.

Effect of epitope-tagged ERβ1 expression on proliferation in MCF 7 human breast cancer cells. When the different cell lines were grown in complete medium containing 5% FBS, induction of ERβ1 but not ERα inhibited the growth of the cells, compared with MCF-7, the control parent cell line. Both β–high (DT+Dox, 1.84 ± 0.24 days, mean ± SEM, n = 6; DT-Dox, 1.23 ± 0.09 days n = 6) and β–low ERβ1 (DT+Dox, 1.84 ± 0.30 days, n = 6; DT-Dox, 1.34 ± 0.10 days, n = 6) over-expressors had significant percent increases in doubling times (% change in DT, Figure 5) in the presence of doxycycline than without (ANOVA, P = 0. 002, n = 6) compared to no significant alterations in the parent (MCF-7: (dT+Dox, 1.11 ± 0.13 days, n = 6; dT-Dox, 1.11 ± 0.13 days) and α–high cells (dT+Dox, 1.32 ± 0.10 days, n = 6; dT-Dox, 1.29 ± 0.10 days). Therefore over-expression of ERβ1 but not ERα can decrease the growth of MCF-7 breast cancer cells in culture.

To determine the effect of over-expression of ERβ1 on the proliferation of MCF 7 cells in the presence and absence of 10 nM E2, the cells were depleted of estrogen as described in the Materials and Methods section, treated with doxycycline for two days, followed by treatment with or without 10 nM E2 in the continued presence of doxycycline. All cell lines including those expressing either tagged ERβ1 or tagged ERα, showed a statistically significant increase in their growth, i.e. a decrease in DT (ANOVA $P < 0.0001$, n = 12-13, followed by t-tests) in response to 10 nM E2 (Figure 6). However,

those cell lines that over-express ERβ1 (either at a low or a high level), had a reduced response to E2 as determined by a reduced % decrease in DT in the presence of E2 (ANOVA P = 0.012, n = 12 –13, followed by t-tests). Both the low and high ERβ1 over-expressors were statistically significantly different from both the parent MCF-7 and the high ERα over-expressor (α-high) controls (Figure 6).

Effect of epitope-tagged ERβ1 expression on the proliferative response of MCF 7 human breast cancer cells to 4-hydroxytamoxifen. An important hypothesis developed from basic studies on the function of ERβ1 using transient transfection and various reporter constructs, is that upregulated expression of ERβ1 may result in tamoxifen resistance. Therefore in the inducible tagged ERβ1 over-expressing cells the responsiveness to 4-hydroxytamoxifen induced growth inhibition was determined. We determined the effect of various concentrations of 4-hydroxytamoxifen on the proliferation rate of β-high, β-low, α-high and MCF-7 parent cell lines. Interestingly, with induction of transgenes (+Dox) a biphasic dose response was observed in the β-high and β-low cells, but not in α-high or MCF-7 control cells (Figure 7A). At low 4-hydroxytamoxifen concentrations ($\leq$ 50 nM) a significant stimulation of proliferation rate was observed (ANOVA, $P < 0.0001$, n = 5). This increase in proliferation rate was higher in β-high compared to β-low cells (ANOVA, $P < 0.0001$, n = 5). At higher concentrations (> 50 nM) growth was inhibited in all cell lines (Figure 7A). Comparing the growth inhibition phase both β-high and β-low were significantly more sensitive to 4-hydroxytamoxifen than either α-high (ANOVA, $P < 0.0001$) or MCF-7 parental cells (ANOVA, $P < 0.0001$). Both β-high and β-low dose-response curves were significantly to the left of both α-high and MCF-7 curves. However, β-low was more sensitive than the β-high so a dose–dependent effect of ERβ1 expression was not observed for growth inhibition. The biphasic growth response to 4-hydroxytamoxifen was also not found in MCF-7 cells inducibly over-expressing the tagged- C-terminally truncated ERβ variant, ERβ2/cx(39). In contrast to the ERβ1, the ERβ2/cx over-expressing MCF-7 cells only showed a growth inhibition in response to 4-hydroxytamoxifen (data not shown). Together these data support the specificity of this biphasic growth response to ERβ1 over-expression in multiple independent clones.

In the absence of transgenes (- Dox, Figure 7B), there was a completed loss of the 4-hydroxytamoxifen induced growth stimulation in β-low and β-high. As well lack of transgene expression (no doxycycline) eliminated any significant differences in sensitivity to tamoxifen growth inhibition between the β-high, β-low and α-high, but not the parental MCF-7 cell line (Figure 7B).

DISCUSSION.

During human breast tumorigenesis and breast cancer progression a marked alteration of estrogen action occurs. Firstly, the responsiveness of ER+ breast tumor cells to the proliferative effect of estrogen is direct (40, 41), in contrast to normal breast epithelial cells where the growth effects of estrogen appear to be indirect (42). Secondly, the expression of ERs is markedly altered between normal breast tissue and breast tumors (15, 17, 18). ERα is up-regulated in ER+ tumors relative to normal breast epithelium whereas current data suggest that ERβ is downregulated in breast tumors compared to normal breast epithelium(15, 17). Since there are both *in vitro* and *in vivo* studies(14, 16, 27, 43) suggesting that ERβ may be a negative modulator of ERα action, it has been speculated that the downregulation of ERβ during breast tumorigenesis removes a modulatory factor of ERα that is important for maintaining normal sensitivity of breast epithelial cells to estrogen action. However, despite the general decreased expression of ERβ in ER+ breast tumors compared to normal tissue, many breast tumors continue to express ERβ and the levels of expression can vary widely amongst tumors(5, 8). And although still controversial, often when ERβ protein is determined, its expression can be correlated with good prognostic markers such as ERα and PR expression(8, 10, 22, 44). This is also consistent with data suggesting that higher ERβ expression is associated with sensitivity to tamoxifen therapy rather than resistance to tamoxifen therapy. However, other data have suggested ERβ association with antiestrogen resistance and poor prognostic markers(21, 45). The development of an inducible ERβ1 expressing MCF7 breast cancer cell line has allowed us to address the functional outcome of altering the relative expression of ERβ1 to ERα in the 'gold standard' model of estrogen/antiestrogen responsive human breast cancer. The development of such breast cancer cell line models

is highly relevant to human breast cancer *in vivo,* since the majority of human breast tumors (~ 59%) have been shown to co-express both ERα and ERβ1(12).

Consistent with studies in which stable over-expression of ERβ1 alone in cell lines was investigated, predominantly nuclear location of the transgene ERβ1 (in our case epitope-tagged) was observed(46). We have isolated various clones of the tetracycline-inducible tagged ERβ1 that under maximal induction conditions express low and high levels of the tagged ERβ1 protein. Using the ligand binding assay we have determined that in low ERβ clones the tetracycline analogue doxycycline increased specific binding of radiolabeled E2 around 200% that of the non-induced (no doxycyclin treatment) and/or non-expressing control cells, while doxycycline treatment of the high ERβ1 expressing clones, resulted in around a 300% increase in specific E2 binding over controls. Since parental MCF7 cells contain predominantly ERα(5), the control and un-induced levels of ligand binding reflect predominantly endogenous ERα levels. Therefore after doxycycline treatment, ERβ was induced to less than or equi-molar levels (estimating from ligand binding assays) to the endogenous ERα in the β–low clones, suggesting that the majority of the ERβ should be in the form of heterodimers with ERα(13) in these clones. However this depends on the kinetics of dimer formation and hence some homodimers of ERα may also exist. In β–high, the tagged ERβ1 was induced to higher levels than β–low clones, suggesting that all the ERα molecules may be heterodimerized with ERβ1, with the possibility that a small population of ERβ1 homodimers may also exist. Although *in vitro,* ERα-ERβ1 heterodimer is apparently favoured (13) it is unclear if this is the case *in vivo.* However, recently it was demonstrated using FRET analysis in living cells that homodimers of each receptor and ERα-ERβ1 heterodimers can occur *in vivo* independently of the presence of ligand(47), supporting the presence in our cell line models of ERβ1 predominantly in the form of ERα-ERβ1 heterodimers. Little, if anything, however is known about the differential function of the various homo- and hetero-dimers of the ER isoforms, although the models described in this study may be useful in addressing such questions.

ERβ1 over-expression alone in ERα negative MDAMB231 human breast cancer cells has resulted in variable results with both growth inhibition(46) and growth stimulation being reported(48). However, when over-expression of ERβ1 in an ERα

positive background has been achieved, more consistent results have been obtained, and over-expression of ERβ1 results in growth inhibition(49, 50). Our results are consistent with these latter data in that ERβ1 but not ERα over-expression results in reduced growth and increased doubling times when cells are grown in complete medium containing FBS and estrogen. In addition, over-expression of ERβ1 but not ERα reduces the magnitude of estrogen induced growth when cells are grown in estrogen depleted medium. These effects of ERβ1 on the proliferation of ERα expressing breast cancer cells are dose-dependent since smaller effects were seen in the low ERβ1 expressing cells compared to the high ERβ1 expressing cells. These data are consistent with the hypothesis that ERβ1 can negatively modulate the activity of ERα when the two ERs are co-expressed. They also suggest that the downregulation of ERβ1 in breast tumors compared to normal breast tissue and therefore the altered relative expression of the two ER is functionally involved in breast tumorigenesis at least for ER+ breast cancer.

However, negative modulation of ERα activity is not a universal effect of over-expression of ERβ1, since in contrast to the effect on E2 induced proliferation, ERβ1 over-expression significantly increased endogenous PR expression, previously considered a specific downstream marker of ERα activity in breast cancer cells. Again in contrast to the growth effects, the increase in PR expression is not ERβ1 specific since over-expression of ERα as seen in α-high cells, similarly increased PR expression. These results support a mechanistic connection associated with the observation *in vivo* where increased ERβ expression determined at the protein level in human breast tumors is often correlated with PR status and expression(7, 8, 22). Therefore ERβ1 regulation of PR expression has physiological and/or pathophysiological relevance. Interestingly, the effect of ER over-expression on PR expression occurs apparently without added ligand (E2) suggesting ligand independence, although further increases in PR expression were seen when the over-expressed ER is bound to E2 (ligand dependent). Indeed since the majority of human breast tumors arise in older, and therefore likely postmenopausal women, one could speculate that PR expression in many breast tumors may reflect the ligand independent activity of the combination of ERα and ERβ1 expressed in the tumor. Alternatively estrogen generated due to aromatase expression by tumor cells as well as the surrounding stroma would also contribute to the steady state PR expression in breast

tumors(51). However, we cannot exclude the possibility that trace amounts of estrogens are still present in the medium and contribute to the increased PR expression. The molecular mechanism by which ERβ1 may regulate PR remains to be determined, although it is due at least in part to increased transcription since steady state PR RNA levels were also increased. However, since ERE half sites adjacent to both AP-1 and Sp1 sites have been previously implicated in the mechanism by which ERα regulates PR expression(52-54), it is speculated that these may also be involved in the mechanism by which ERβ1 can regulated PR expression. However, if the AP-1 site is important in this regard, this would be an example of E2 activation of ERβ1 being involved in increased rather than decreased transcription of an AP-1 regulated estrogen responsive promoter(20). We cannot however, exclude effects on PR mRNA stability at this stage.

The involvement of ERβ1 in tamoxifen sensitivity is controversial, and in a previous study when inducible over-expression of ERβ1 was investigated after stable expression in T-47D breast cancer cells, which also express endogenous ERα, only growth inhibition due to tamoxifen was observed(50). No stimulation of growth was reported. However, full dose-response analyses covering low and high concentrations of 4-hydroxytamoxifen were not undertaken(50). To the best of our knowledge, our data are the first to show any effect of altered ERβ1 expression on tamoxifen regulated proliferation in breast cancer cells. In the presence of estrogen replete (phenol-red and FBS containing) medium, a biphasic growth response to 4-hydroxytamoxifen, was observed only in ERβ1 over-expressing cells. Neither the parental control nor the ERα over-expressing cells showed a biphasic response. The biphasic growth response was lost when ERβ1 over-expression was not induced (no doxycycline), and was not present when the ERβ variant, ERβ2–cx, was over-expressed under the same conditions.

The growth stimulatory response seen at low doses of 4-hydroxytamoxifen, was only seen when ERβ1 was over-expressed, its magnitude was correlated with the level of ERβ1 and was completely eliminated in the absence of ERβ1 induction (i.e no Dox). These data suggest that ERβ1 expression was functionally involved in the 4-hydroxytamoxifen stimulation of proliferation seen at low doses of the antiestrogen. When considering the growth inhibition phase, doxycycline treatment of all cell lines except α-high significantly moved the dose-response curves to the left, compared to the

no doxycycline treatment i.e no induction of expression of the transgene. This suggests that a higher ERβ1 but not ERα increased sensitivity to the growth inhibitory effects of 4-hydroxytamoxifen. However, removal of doxycycline also reduced the sensitivity of the MCF-7 control cells to 4-hydroxytamoxifen, suggesting that clonal variation may also in part contribute to, but not entirely explain, the differences seen in sensitivity to the growth inhibitory, but not the growth stimulatory effects of tamoxifen in these cell lines. In particular, there was no evidence that ERβ1 over-expression resulted in a shift of the dose response curve to the right of any of the control cells, which supports the conclusion that acute over–expression of ERβ1 is not associated with resistance or decreased sensitivity to tamoxifen. Overall these data support the hypothesis that increased expression of ERβ1 but not over-expression of ERα in the presence of endogenous expression of ERα increases the sensitivity of human breast cancer cells to the growth modulatory effects of tamoxifen.

Our results showing that ERβ1 over-expressing cells display an increased sensitivity to tamoxifen, is consistent with increased expression being associated with tamoxifen sensitivity and not resistance *in vivo* in breast cancer patients(7, 22). The stimulation of proliferation due to tamoxifen seen at low doses, is interesting and we would argue that rather than be interpreted as representing a potential form of tamoxifen resistance(55), since it only occurs a very low concentrations, (tamoxifen resistance occurs *in vivo* at varying periods of time following or during high doses of tamoxifen) is likely to resemble and/or be a model of "tamoxifen flare" that has been observed clinically(56-58). In this condition an apparent worsening of the disease is seen in some patients in the first few weeks of starting tamoxifen treatment. Likely before sufficiently high steady state concentrations of tamoxifen and its active metabolites have accumulated. This is often a favorable sign for responsiveness to tamoxifen therapy since in the cases where tamoxifen therapy was continued, subsequent tumor regression often occurred(58, 59). Interestingly, only one other breast cancer cell culture model of "tamoxifen flare" has been documented previously(60). In this case the cell line was T-47D and several labs have often described a relatively higher natural level of ERβ expression in T47-D cells compared to other ER+ breast cancer cell lines(6, 61). The mechanism by which ERβ1may increase the sensitivity of breast cancer cells to

tamoxifen is unknown, but the higher relative binding affinity of ERβ1 compared to ERα for 4-hydroxytamoxifen may contribute in part(62).

In conclusion our data using inducible, ERβ1 over-expressing MCF-7 cells do not support the hypothesis that up-regulation of ERβ1 in the presence of ERα is a mechanism of antiestrogen resistance per se. Although we cannot exclude a role of over-expression of ERβin resistance in combination with other changes such as altered cofactor expression and/or activity. As well our data support the hypothesis that ERβ1 can be a negative modulator of ERα mediated growth response since over-expression of ERβ in a dose-dependent fashion inhibits the magnitude of the E2 induced growth response in ER+ human breast cancer cells. However, over-expression of ERβ1 does not inhibit or reduce all ERαregulated activities since endogenous PR expression is not decreased in these cells. Indeed ERβ1 upregulates the endogenous expression of PR in possibly both a ligand independent and dependent fashion. The parallels seen with our over-expressing ERβ1 MCF7 cell lines and some *in vivo* studies showing a relationship between PR and ERβexpression as well as ERβ expression and tamoxifen sensitivity of breast cancer patients suggest that these models are relevant and useful for dissecting the role of ERβ1 expression in ER+ breast cancer.

ACKNOWLEDGEMENTS.

We thank Christina Walker for her technical assistance.

Table 1. Estrogen receptor levels measured by ligand binding assay of doxycycline induced and uninduced MCF-7 cell lines.

Clone	**Sites per cell** (+ Dox; 48 hrs) **(mean ± SD)**	**Sites per cell** (No Dox) **(mean ± SD)**
MCF-7 (parent)	33910 ± 5831 (n = 3)	34150 ± 11030 (n = 3)
MCF-7-V (vector alone)	42260 ± 5445 (n =2)	45190 ± 12110 (n = 2)
MCF-7- β-low (ERβ1)	70360 ± 9004 (n =3)	38120 ± 11210 (n = 3)
MCF-7-β-high (ERβ1)	98580 ± 12350 (n = 3)	37280 ± 22850 (n = 3)
MCF-7-α-high (ERα)	108700 ± 30630 (n = 3)	49400 ± 19180 (n = 3)

Legends to Figures.

Figure 1.

A. Western blot of cell extracts from cell lines grown in 5%CS medium treated (+) or not treated (-) with doxycycline for 48 hours. ERβ1 transgene was visualized using anti-Xpress antibody as described in Materials and Methods. MCF-7 parent cells stably expressing rTA Tet-On transcription factor (29); V = Vector alone transfected MCF7 cells ; β–low = Xpress-tagged ERβ1 low expressing MCF-7 clone; β–high = Xpress-tagged ERβ1 high expressing MCF-7 clone; α–high = GFP-tagged ERα high expressing MCF-7 clone.

B. Same blot as in A, stripped and visualized using anti-ERα antibody as described in Materials and Methods (note the larger GFP- ERα and the smaller approx. 66 kDa endogenously expressed ERα).

C. Same blot as in A, stripped and visualized using anti-tubulin antibody as described in Materials and Methods

Figure 2.

Immunofluorescent detection of Xpress-tagged ERβ1 in induced (+Dox) and uninduced (-Dox) β–low and control parental MCF-7 cells.

Figure 3.

A. Effect of estrogen and antiestrogens on detection of tagged ERβ1 in MCF-7 cells in estrogen depleted medium. Upper panel visualized with anti-Xpressed antibody; lower panel visualized with anti-actin antibody. E2 = treated with 10 nM estradiol-17β; C = vehicle (ethanol) treated control; T = treated with 500 nM 4-hydroxytamoxifen; I = treated with 500 nM ICI 182780. β–low = Low ERβ1 expressor +Dox for 48 hrs followed by ± ligands for 24 hrs;

B. Effect of E2 and antiestrogens on endogenous ERα (top panel) and PR (middle panel) expression in the presence of over-expression of ERβ1 (+Dox) or no over-expression of ERβ1 (-Dox). Bottom panel visualized with anti-tubulin antibody. The same blot was

used for ERα was stripped and visulaized with anti-PR antibody and then anti-tubulin antibody as described in Materials and Methods.

The results shown in this figure are from β-low treated (+Dox) or not treated (-Dox) for 48 hrs, followed by treatment ± ligand for 24 hrs, as described above.

Figure 4.

A. Effect of ER transgene expression (+ Dox for 48 hours) on endogenous PR expression in cells grown under estrogen depleted conditions. Western blot visualized with anti-PR antibody. MCF-7 parent cells stably expressing rTA Tet-On transcription factor (29); V = Vector alone transfected MCF7 cells ; β–low = Xpress-tagged ERβ1 low expressing MCF-7 clone; β–high = Xpress-tagged ERβ1 high expressing MCF-7 clone; α–high = GFP-tagged ERα high expressing MCF-7 clone.

B. Same blot as in A, stripped and visualized with anti-tubulin antibody.

Figure 5. Effect of ER transgene induction on proliferation of cells in 5%CM medium. Uninduced cells were set up at 10^4 cells per well in 5%CM, followed by ± Dox (2 μg/ml). Media was changed every 2 days. Cells were harvested and counted electronically at various times after treatment (usually day 4 and day 8). Doubling times were calculated as described in Materials and Methods. The change in DT between no transgene induction (-Dox) and induction of transgene (+Dox) was calculated as a %. The mean +/- SEM of 6 independent experiments is shown. ANOVA (P = 0. 002) followed by t- testing indicates that the increase in DT as a percentage seen in the β-low over-expressor and the β-high over-expressor is significantly different from that of the control parent cell line, MCF 7 (P = 0.022 and P = 0.004, respectively); the over-expressing α-high cell line is not significantly difference from the control parent cell line, MCF 7; the β-low and the β-high over-expressors are significantly different from α-high (P = 0.03 and P = 0.005, respectively).

Figure 6. Effect of ER transgene over-expression on growth of cells in response to estrogen. Cells were depleted of estrogen and set up on day zero at 5 X 10^4 cells per well in 5%CS containing medium, treated for 48 hours with doxycycline and then treated with

E2 (10 nM) or vehicle alone (ethanol). Cells were harvested and counted at the various times after E2 and medium was changed every 2 days. Doubling times (DT) were calculated as described in Materials and Methods. The % change in DT due to E2 treatment was calculated as DT-E2/DT-Vehicle alone X 100. The mean +/- SEM of n = 12 (parent MCF7 cells, and ERα over-expressing cells = α-high) and n = 13 (ERβ1 low over-expressor = β-low and ERβ1 high over-expressor = β-high) independent experiments is shown. Significant differences were found for all cell lines in the % decrease DT due to E2 treatment; ANOVA, $P < 0.0001$, followed by t-testing). As well differences between cell lines in % decreased DT due to E2 were found (ANOVA, P = 0.012) where β-low is significantly less than both MCF-7 and α-high (*, P = 0.033, P = 0.049, respectively); similar comparisons for β-high were **, P = 0.002, P = 0.003). No significant differences were seen between MCF-7 and α-high. DTs (mean ± SEM days, n = 13-12) in presence of E2: MCF-7, 1.75 ± 0.1 days; α-high, 1.62 ± 0.1 days; β-low , 1.83 ± 0.12 days; β-high, 2.04 ± 0.12 days. DTs (mean ± SEM days, n = 13-12) in absence of E2: MCF-7, 3.73 ± 0.81 days; α-high, 2.82 ± 0.36 days; β-low, 2.33 ± 0.19 days; β-high, 2.24 ± 0.13 days.

Figure 7. A. Effect of ER over-expression (+Dox) on 4-hydroxytamoxifen sensitivity in MCF-7 breast cancer cells. Cells were grown in 5%CM and set up on day zero at 10^4 cells per well in 5%CM containing medium, treated for 48 hours with doxycycline and then various concentrations of 4-hydroxytamoxifen or vehicle alone. Cells were harvested and counted electronically at various times after antiestrogen treatment. Doubling times and proliferation rates were calculated as described under Materials and Methods, and the mean +/- SEM of n = 5 (for 4-hydroxytamoxifen concentrations 1-75 nM) and n = 3 (for 4-hydroxytamoxifen concentrations 100-1000 nM) independent experiments with results expressed as proliferation rate % of control (ethanol alone treated cells). Medium was changed every 2 days.

Filled squares are parental control cells, MCF-7 clone 89 (rTA, Tet-on transcription factor expressor only).

Open squares are α-high overexpressing MCF-7 cells.

Filled circles are β–low overexpressing MCF-7 cells.

Filled triangles are β–high overexpressing MCF-7 cells.

B. 4-hydroxytamoxifen sensitivity in MCF-7 breast cancer cell lines in absence of tagged ER-over-expression (- Dox). Cells were grown as described above except that they were not treated with doxycycline. Cells were harvested and counted electronically at various times after antiestrogen treatment. Doubling times and proliferation rates were calculated as described under Materials and Methods, and the mean +/- SEM of n = 3 independent experiments with results expressed as proliferation rate % of control (ethanol alone treated cells). Medium was changed every 2 days.

Filled squares are parental control cells, MCF-7 (rTA, Tet-on transcription factor expressor only).

Open squares are α–high overexpressing MCF-7 cells.

Filled circles are β–low overexpressing MCF-7 cells.

Filled triangles are β–high overexpressing MCF-7 cells.

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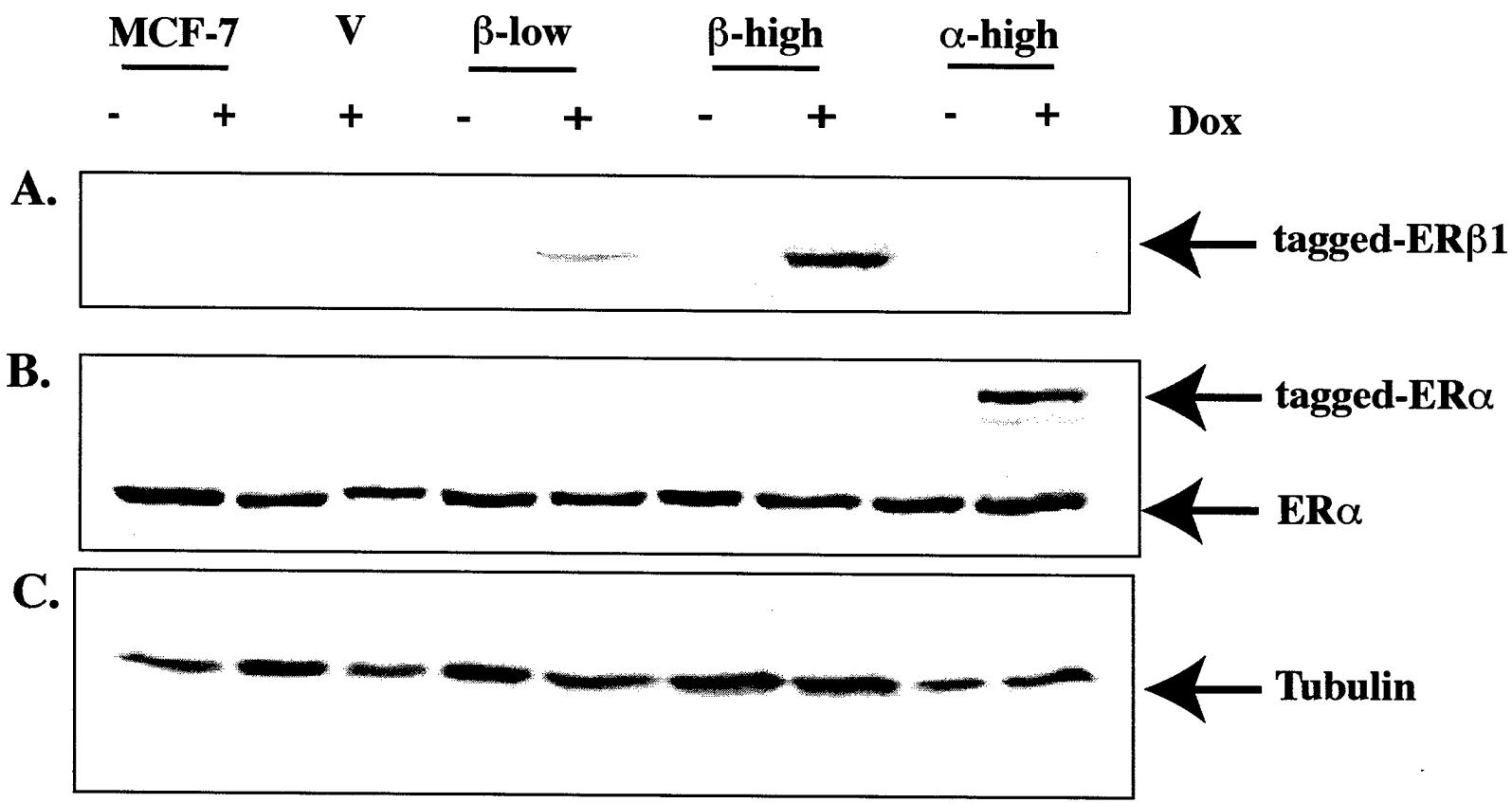
Figure 1.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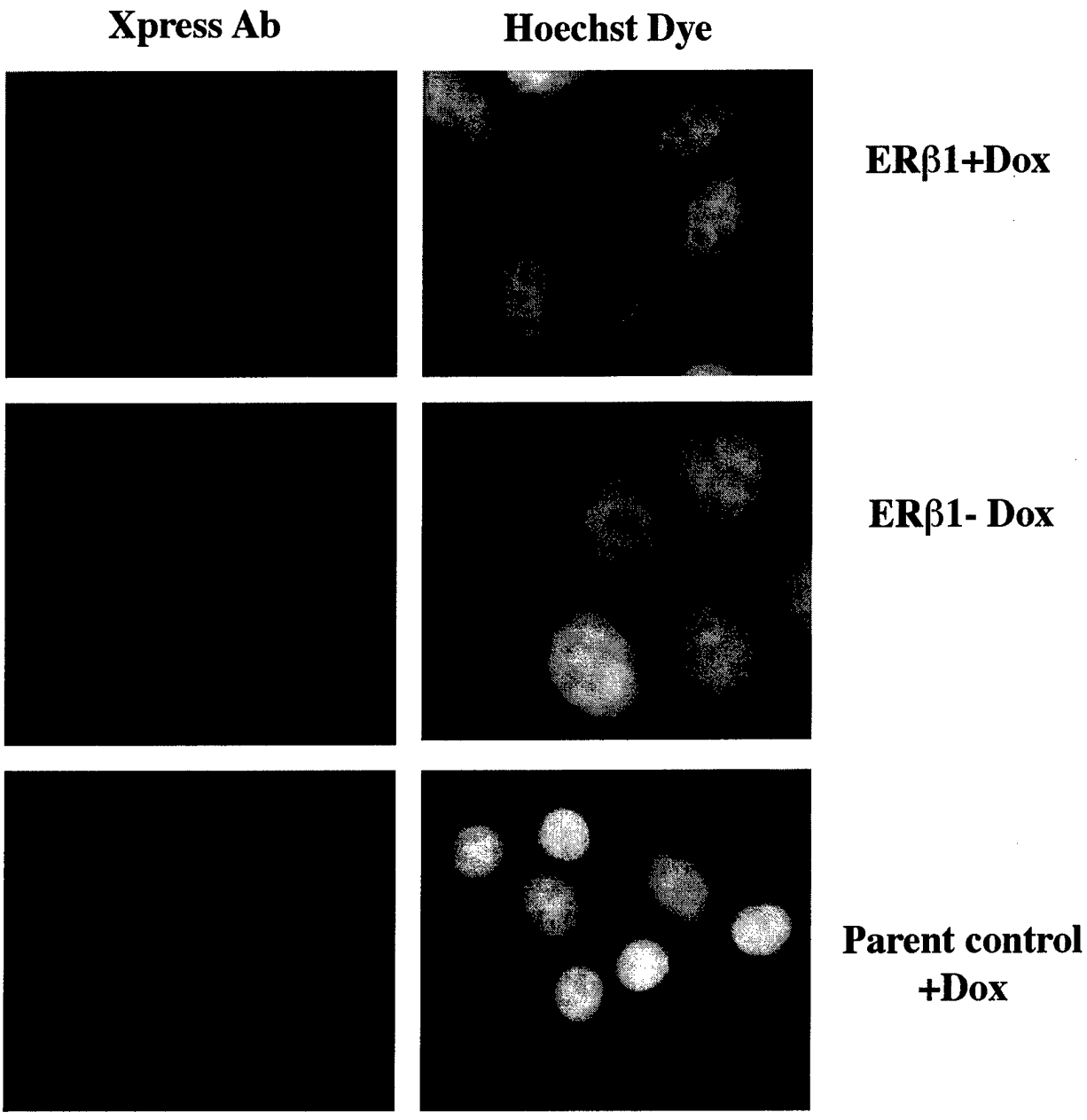
Tubulin

Figure 2



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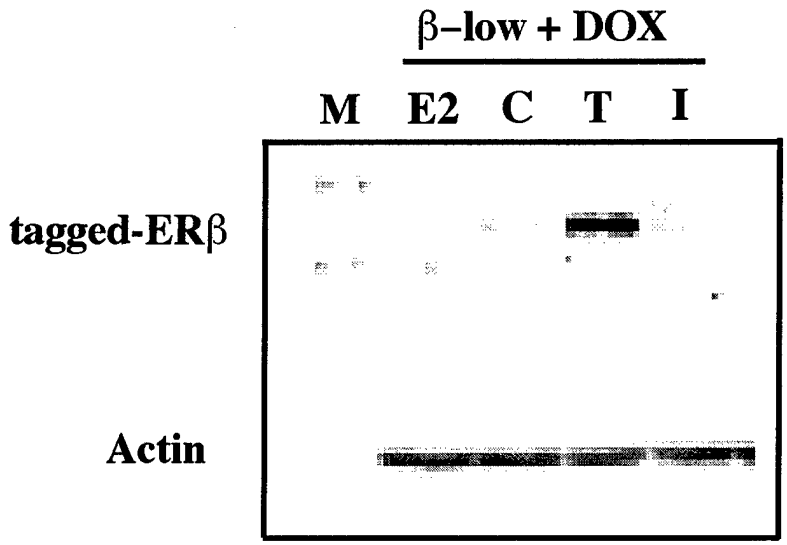
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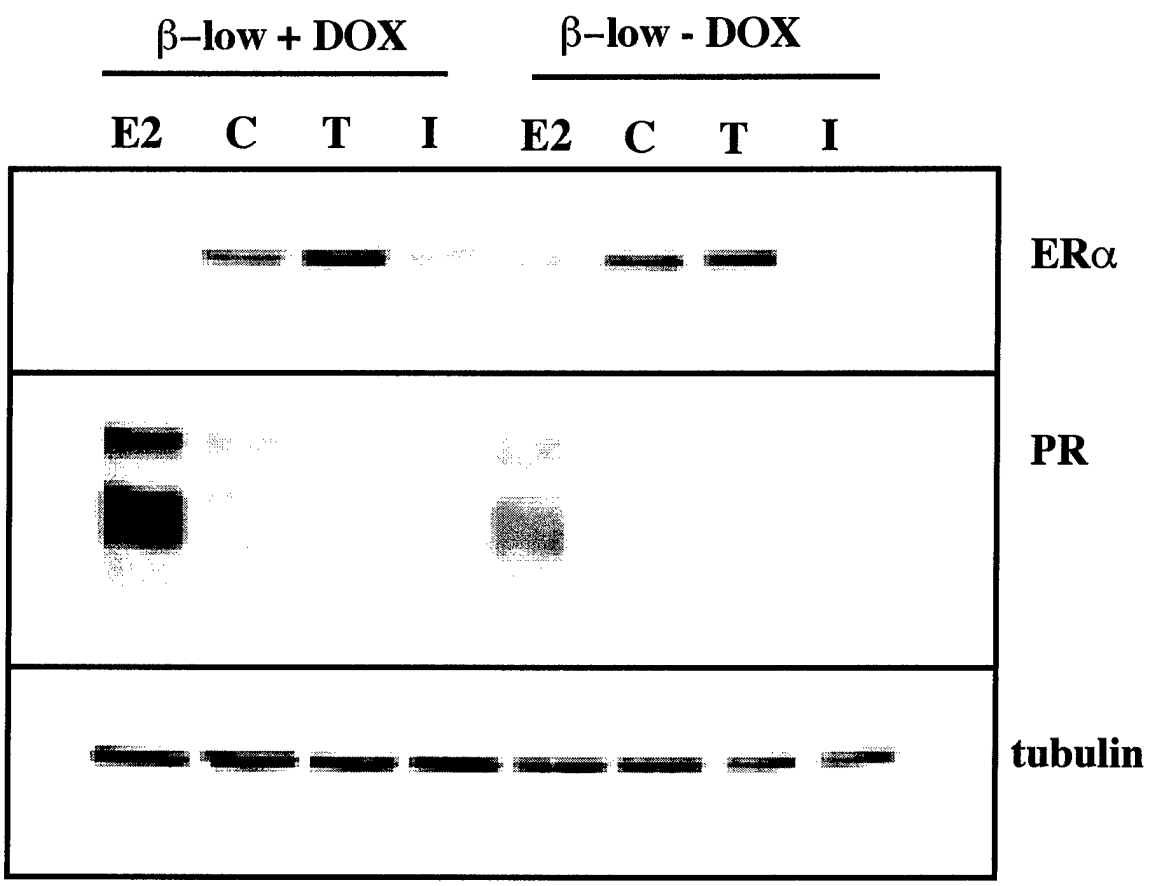
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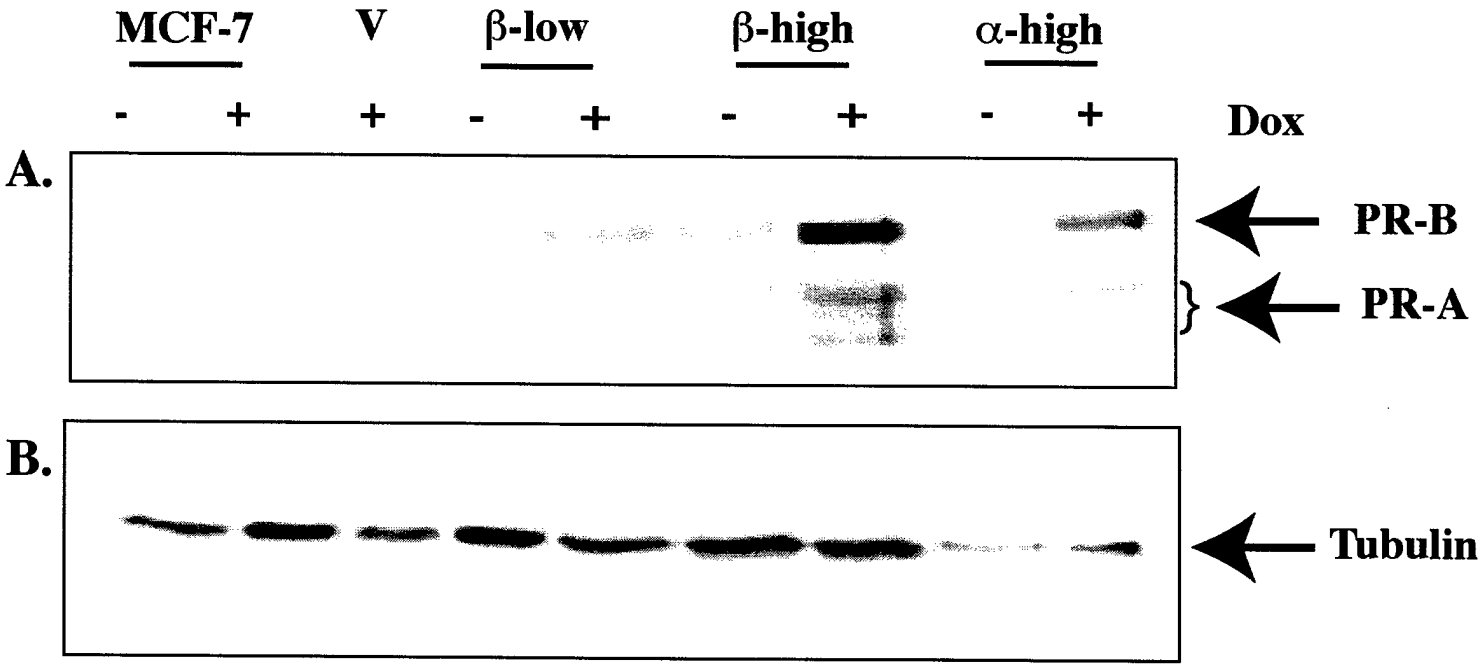
Figure 4.



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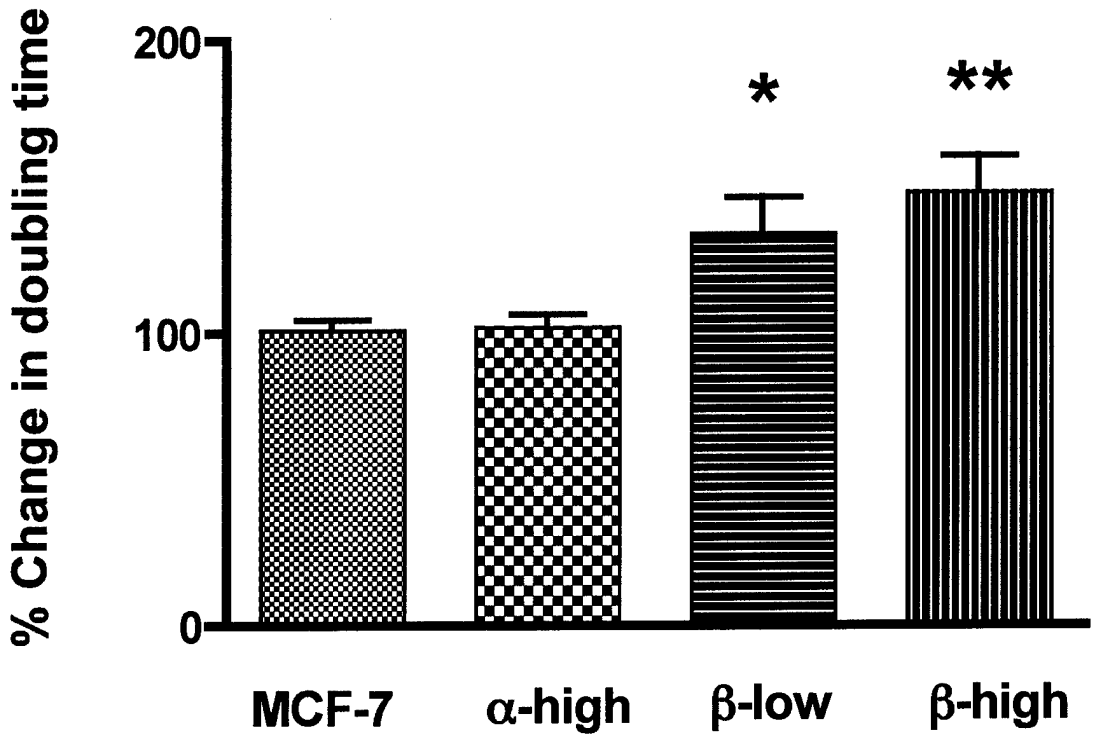

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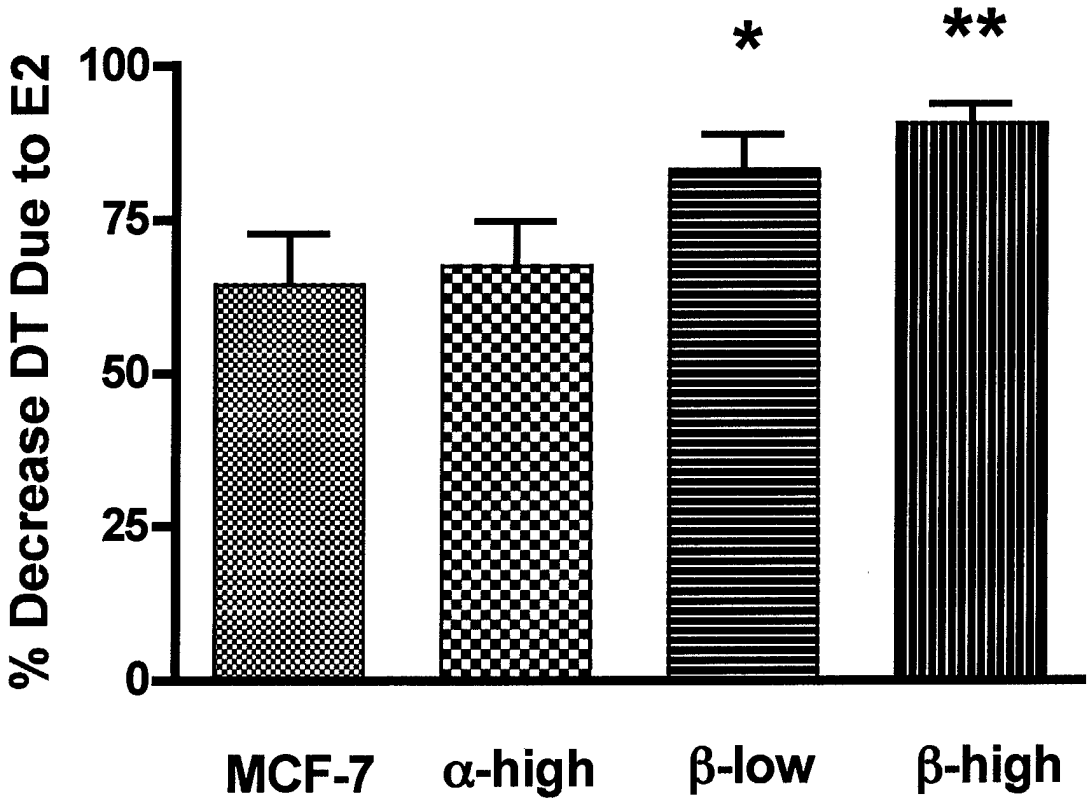
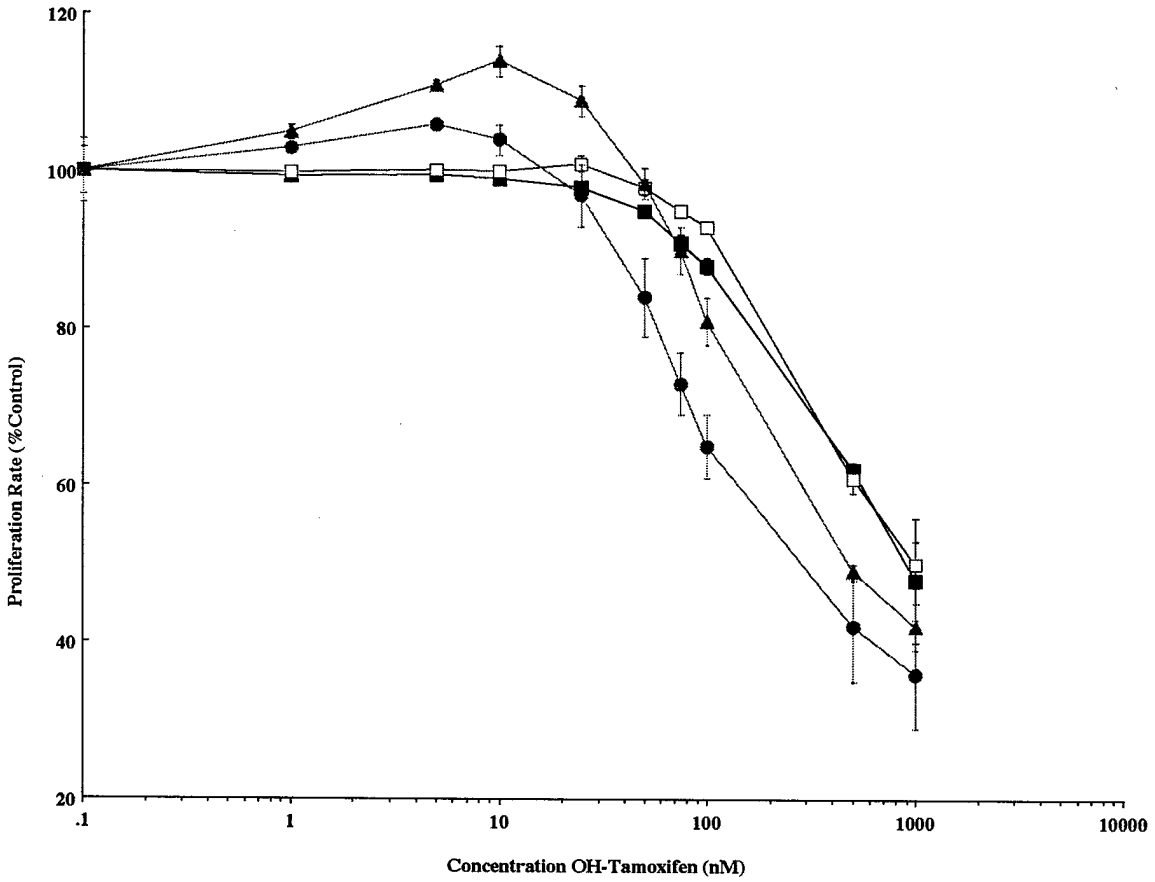


Figure 7A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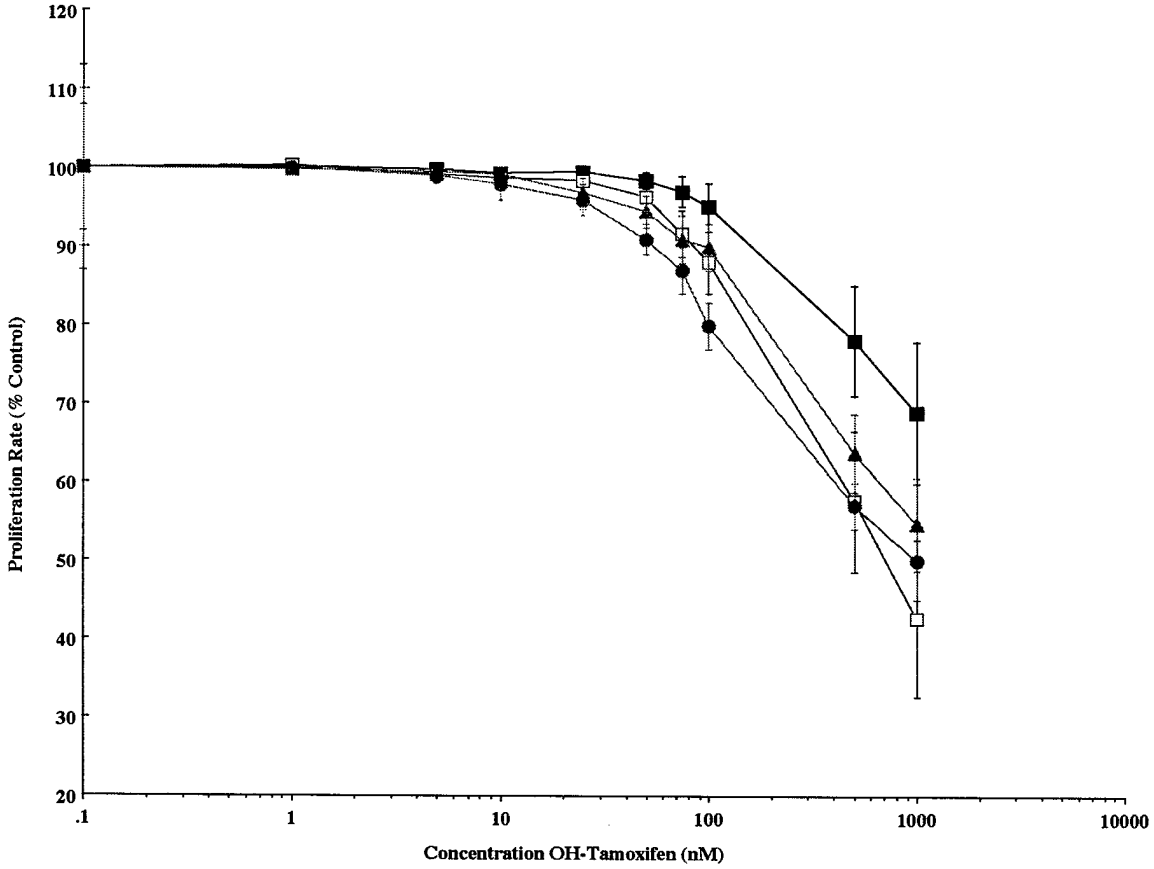
Concentration OH-Tamoxifen (nM)

Figure 7B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Proliferation Rate (% Control)
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Concentration OH-Tamoxifen (nM)

Appendix 10 Abstract #5597. AACR meeting 2004

Abstract Number: 5597

Psoriasin; A possible downstream marker of estrogen receptor β1 activity in human breast cancer cells

Anthony Lewis, Ethan Emberley, Baocheng Peng, Marcello Venditti, Etienne Leygue, Angella Kemp, Jim Davie, Robert Shiu, Peter Watson, Leigh Murphy.

University of Manitoba, Winnipeg, MB, Canada.

A role for estrogen receptor (ER)β in breast cancer is becoming clear, and a downstream marker of ERβ activity in breast cancer is required to further investigate the role of ER. During breast tumorgenesis levels of S100A7 (psoriasin) gene expression rise from undetectable in normal, to high levels in DCIS and generally decline in invasive breast cancer. However, our group has previously identified that maintenance of psoriasin expression in invasive breast cancer is an indicator of poor patient survival. We have observed that in the ER positive breast cancer cell lines MCF7 and ZR75, psoriasin mRNA can be induced in response to 10nM estradiol (E2), peaking around 24 to 48 hours after treatment. As psoriasin expression is more commonly observed in ERα negative tumors in vivo, we hypothesize that psoriasin may be regulated by ERβ1, which is also expressed in MCF7 cells at low levels. Utilizing MCF7 clones stably transfected with tet-inducible ERα or ERβ, we have observed upregulation of psoriasin RNA and protein in ER_1 over-expressing clones in response to E2 treatment. Neither the over-expressing ERα clone nor the truncated non-ligand binding ERβ2 control clones showed this upregulation. Treatment of ERβ1 clones with excess anti-estrogens 4-OH-tamoxifin or ICI 182,780 abolished the observed E2 induced psoriasin protein upregulation. Using different ERβ1 clones, which produced different levels of ERβ1, we observed a dose-dependent increase of psoriasin protein in response to increasing levels of ERβ1 protein, peaking at 72 hours post-treatment. To further clarify upregulation of psoriasin as a possible downstream marker of ERβ1 activity, we have studied the effects of ER-isoform specific ligands, gifts from Astrazeneca. Our experiments show that treatment of induced ERβ1 clones with 10nM ERβ1-specific ligand upregulated psoriasin protein. Conversely, the same cells showed no upregulation of psoriasin in response to 10nM treatment with the ERβ-specific ligand. As increasing psoriasin RNA levels peak between 24 and 48 hours, while protein levels peak at 72 hours post treatment, we have further investigated the observed delay in protein production. Utilizing 50μM cycloheximide (CHX) treatment of ERβ1 clones prior to E2 treatment, we observed that CHX abolishes the observed upregulation of psoriasin RNA. These data indicate that psoriasin up-regulation in response to E2 is indirect, requires protein synthesis and may involve another protein. Thus the data presented support the hypothesis that psoriasin may be the first specific downstream marker of ERβ1 activity in breast cancer cells, and that this effect is accomplished through an indirect mechanism.

Presenter: Anthony Lewis

Affiliation: University of Manitoba, Winnipeg, MB, Canada; E-mail: umlewisa@cc.umanitoba.ca

 Citation information: Proceedings of the AACR, Volume 45, March 2004.

Appendix 11 Biochem Biophys Res Com. 2003. 301: 509-515

Available online at www.sciencedirect.com
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Identification of new human coding steroid receptor RNA activator isoforms☆

Ethan Emberley, Guo-Jian Huang, Mohammad K. Hamedani, Anna Czosnek, Djiae Ali, Allen Grolla, Biao Lu, Peter H. Watson, Leigh C. Murphy, and Etienne Leygue*

Department of Biochemistry and Medical Genetics, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3EOW3

Received 19 December 2002

Abstract

SRA is a steroid receptor co-activator which acts as a functional RNA and is classified as belonging to the growing family of functional non-coding RNAs. None of the different SRA transcripts described to date encode a detectable SRA protein following in vitro and in vivo translation experiments. We have identified three new SRA-RNA isoforms differing mainly from the originally cloned SRA by an extended 5′ extremity. These long SRA isoforms, able to encode a stable protein in vitro, led to the production in vivo of a nuclear protein when transfected into the MCF-7 human breast cancer cell line. Reverse-transcription polymerase chain reaction and Western blot analysis of RNA and protein extracts from different breast cancer cell lines confirmed the presence of endogenous coding SRA isoforms and their corresponding proteins. Our results demonstrate that full-length SRA-RNAs likely to encode stable proteins are widely expressed in breast cancer cell lines.

Keywords: SRA; Steroid receptor co-activator; Normal and tumor human breast tissue; Polymorphism; Tumorigenesis; PCR

Endogenous steroid hormones such as estrogen, progesterone, and androgen regulate the growth and the development of several organs and tissues including brain, bones, and reproductive organs such as uterus, testis, and breast. Steroid hormone actions are mainly mediated through specific receptors that belong to the steroid/thyroid/retinoic acid receptor super-family and act as ligand-dependent transcription factors [1–3]. The transcription of target genes by hormone-liganded receptors depends upon interactions between these receptors and several members of a complex co-regulator population (see [2] and references herein). Among co-regulators, co-activators are proteins defined as enhancing hormone induced transactivation without altering basal transcriptional activity and as reversing squelching between different receptors when overexpressed [3]. To an already long list of nuclear receptor co-activators, Lanz et al. [4] recently added SRA, a steroid receptor specific activator that differs from other co-activators in two main features. First, SRA co-activates steroid receptors as an RNA and not as a protein. These authors were unsuccessful in trying to generate in vitro or in vivo stable SRA protein but demonstrated that SRA-RNA existed in a ribonucleoprotein complex activating steroid receptor induced transcription in the absence of a translated SRA protein [4]. Second, as opposed to most positive co-regulators that interact with and co-activate both class I and class II nuclear receptors, SRA appears to be specific for steroid receptors. SRA expression is modified during breast tumorigenesis and breast tumor progression and we have suggested that this co-activator could be involved in the molecular mechanisms underlying these events [5,6]. More recently, it has been shown that antisense oligonucleotides can be used to decrease endogenous SRA-RNA [7] and that this RNA interacts with other proteins such as Sharp and RNA-binding DEAD-box p72/p68 proteins to modulate steroid

☆ *Abbreviations:* SRA, steroid receptor RNA activator; ER, estrogen receptor; PR, progesterone receptor.

* Corresponding author. Fax: +204-789-3900.
E-mail address: eleygue@cc.umanitoba.ca (E. Leygue).

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doi:10.1016/S0006-291X(02)03070-X

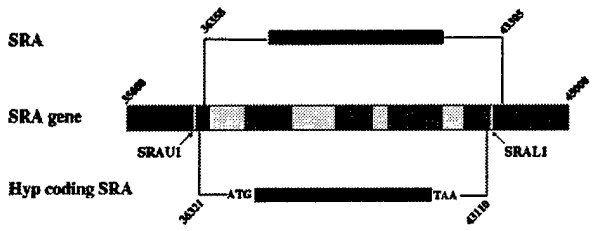

Fig. 1. Schematic representation of SRA–mRNA and gene structure. Human SRA isoforms identified to date differ slightly in their 5′ and 3′ terminal regions but a common nucleotide alignment sequence can be generated. The only SRA sequence entered in GenBank (AF092038) is fully contained within 10,000 bp of Bac 5 genomic sequence (AC005214). Dark gray boxes correspond to exons numbered appropriately. Numbers indicate positions relative to this latter sequence. Hyp coding SRA: hypothetical coding SRA sequence found using Gene Finder. Positions of SRAU1 and SRAL1 primers are indicated by arrows. Light gray boxes are introns.

receptor activity [8,9]. Overall, SRA is now considered as a member of the expanding family of functional non-coding RNAs [10].

The first published sequence for SRA (GenBank AF092038) was fully contained in a genomic sequence of chromosome 5 (GenBank AC005214), within five separated exon-like regions (Fig. 1). Within this latter clone we have found, using Gene finder (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html), the sequence of a hypothetical mRNA which could encode a 236 amino-acid protein that corresponds exactly to the first published SRA sequence, except for an additional 37 nucleotides in the 5′ region (Fig. 1). This new 5′ region now contains an AUG start codon encoding the first methionine of a novel, putative 236 amino-acid SRA protein in contrast to the previously predicted 162 amino-acids which were unstable. We have investigated the expression of this hypothetical mRNA and its corresponding protein in breast cancer cell lines.

Materials and methods

Human breast tissues and cell lines

Breast epithelial cell lines (BT-20, MDA-MB-468, MDA-MB-231, MCF10AT1, MCF10A1, MCF10AT3B, ZR-75, T47D, T5, MCF7, and HBL100) were grown, harvested, and cell pellets were stored at −70 °C, as previously described [11]. Total RNA and DNA were extracted from frozen normal breast tissue sections (obtained from the Manitoba Breast Tissue Bank) and cell pellets using Trizol reagent (Gibco-BRL, Grand Island, NY) according to the manufacturer's instructions [12]. Total proteins were extracted from frozen cell pellets as previously described [13].

Primers and RT-PCR conditions

Detection and cloning of the hypothetical coding SRA-RNAs. Primers used consisted of SRAU1 primer (5′-TCCTTTGGTGCC TTGTGAC-3′; sense; positions 36,132–36,150, GenBank Accession No. AC005214) and SRAL1 primer (5′-AGTCTGGGGAACCGAGG AT-3′; antisense; positions 43,128–43,110, GenBank Accession No. AC005214). One microgram of total RNA was reverse transcribed in a final volume of 25 μl using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and random hexamers as previously described [13,14]. The PCR Optimizer Kit (Invitrogen, Carlsbad, CA) was used to set up optimal RT-PCR amplification conditions. One microliter of RT mixture was amplified in a final volume of 30 μl, in the presence of 60 mM Tris–HCl (pH 8.5), 15 mM $[NH_4]_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng/μl of each primer, and 1 U Taq DNA polymerase (Gibco-BRL). Each PCR consisted of a 5 min pre-incubation step at 94 °C followed by 30 cycles of amplification (30 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C). PCR products were sub-cloned using TOPO TA cloning kit (for sequencing and in vitro translation experiments) and pcDNA3.1/V5-His TOPO TA expression kit (for stable expression) according to the manufacturer's instruction (Invitrogen, Carlsbad, CA) and sequenced as previously described [14].

Detection of SRA isoform 3. Primers used consisted of SRAU2 primer (5′-GGGCCTCCACCTCCTTCAAGTA-3′; sense; positions 41664–41685, GenBank Accession No. AC005214), SRAL2 primer (5′-GCAGTCTTCCAATGCCTG-3′; antisense; positions 41813–41796, GenBank Accession No. AC005214), and SRAL-**GTCG** primer (5′-C ACATCCTCCATCA**GTCG**-3′; antisense; positions 41780–41767 which is specific for SRA isoform 3, GenBank Accession No. AC005214 plus **GTCG** sequence). Radioactive PCR amplifications were performed in the presence of [α-^{32}P]dCTP and SRAU2/SRAL2 or SRAU2/SRAL-**GTCG** primers and PCR products were separated on poly-acrylamide gels as previously described [15]. Following electrophoresis, the gels were dried and exposed 30 min to a Molecular ImagerTM-FX Imaging screen (Bio-Rad, Hercules, CA). Exposed screen was then scanned using a Molecular ImagerTM-FX (Bio-Rad, Hercules, CA). As positive control, we amplified 10 ng of sequenced plasmids previously shown to correspond to SRAcod1, SRAcod2, and SRAcod3 inserted.

In vitro synthesis of SRA protein

[^{35}S]Methionine-labeled SRA proteins were generated in vitro using Reticulocyte Lysate coupled transcription/translation reactions by the TnT System (Promega, Madison, WI) according to the manufacturer's instructions using expression plasmids corresponding to SRAcod1, SRAcod2, or SRAcod3 inserts. Lysates were then subjected to SDS–PAGE, after which gels were dried, and [^{35}S]methionine-labeled protein bands were visualized by exposing overnight to a Molecular Imager-FX Imaging screen (Bio-Rad, Hercules, CA) and subsequently scanned using a Molecular Imager-FX (Bio-Rad, Hercules, CA).

Stable transfection and immunofluorescence. MCF7-SRA1 and MCF7-SRAN cells were engineered by stably transfecting MCF-7 breast cancer cells with pcDNA3.1/V5-His TOPO TA vector containing the full SRA1 coding sequence (between primers SRAU1 and SRA-L3, see Fig. 2) either in frame with a C-terminal V5-His Tag (SRA1) or in an inverse orientation (SRAN), using Lipofectamine reagent according to the manufacturer's protocol (Life Technologies, Gaithersburg, MD). To insure the retention of the transgene, transfected cells are maintained in the presence of Geneticin (Gibco-BRL, Grand Island, NY, 500 μg/ml). MCF7-SRA1 and MCF7-SRAN cells grown on 4-well slides were fixed in freshly prepared 3.7% paraformaldehyde (30 min at 37 °C). Following fixation, slides were blocked in 1% FBS–PBS (overnight, 4 °C) and incubated with anti-V5 (Invitrogen, Carlsbad, CA, 1:750 dilution) primary mouse antiserum (1 h, room temperature). Slides were thoroughly washed in PBS followed by addition of the Cy3-conjugated goat anti-mouse secondary antiserum (Jackson Immuno-Research, 1 h, 37 °C, 1:10,000 dilution). Slides were counterstained with Hoechst (1 mg/ml, 30 min), washed extensively with PBS, mounted in FluorSave mounting reagent (Calbiochem), and visualized using an E1000 Nikon microscope (UV-2A or G-2A filters) with epifluorescence illumination and a DXM 1200 Nikon camera. All images were processed using Act-1 (Nikon) and Adobe Photoshop software.

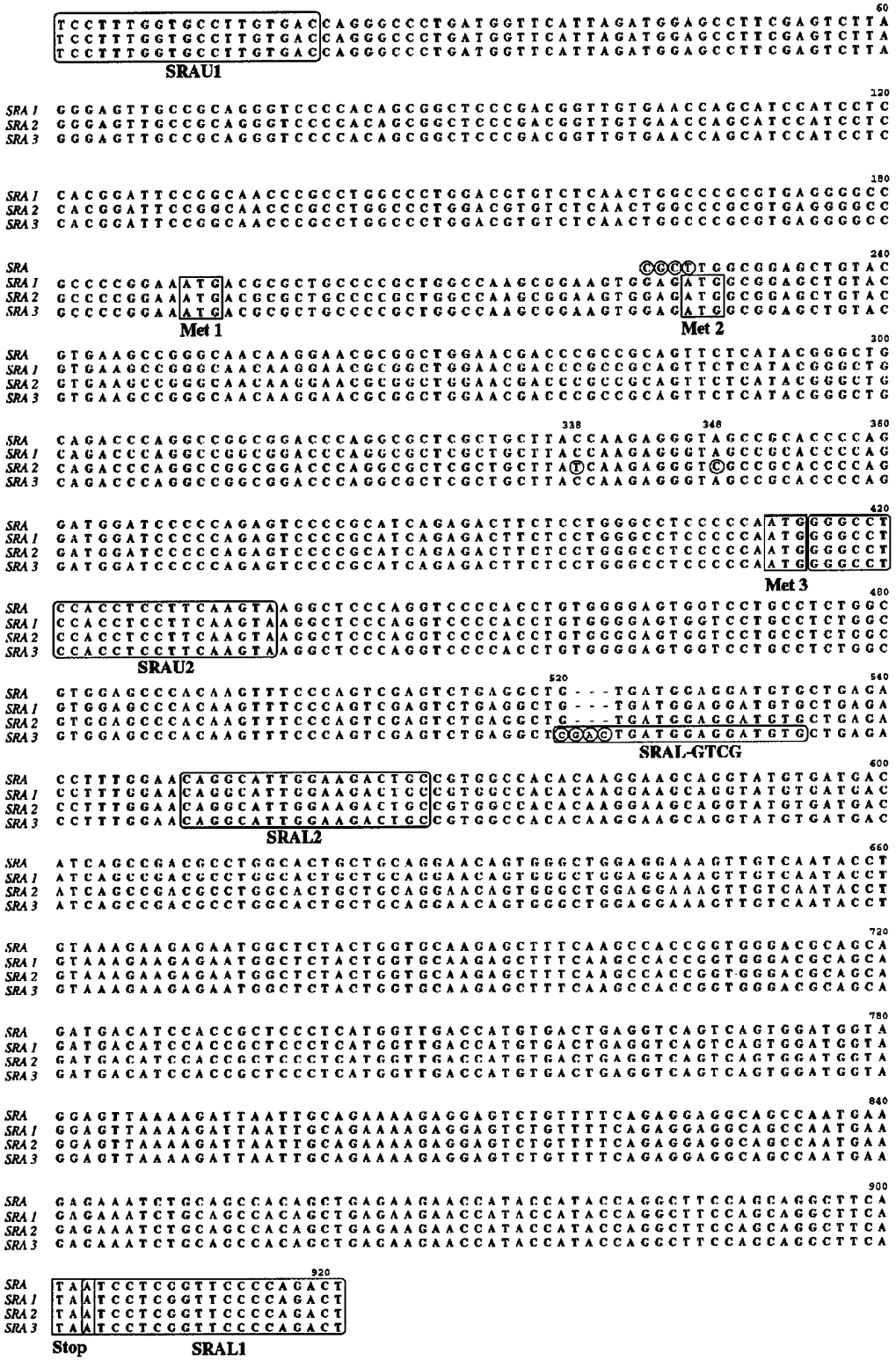

Fig. 2. Alignment of SRA isoforms and primer positions. Previously (4) cloned SRA sequence (AF092038) is aligned with new SRA isoforms: SRA1 (AF293024), SRA2 (AF293025), and SRA3 (AF293026). Differences between sequences are circled. Positions of two new putative starting ATG codons are indicated (Met 1 and Met 2), together with that of the initially predicted starting codon (Met 3, [4]) and the common stop codon. The positions of SRAU1, SRAU2, SRAL-GTCG, SRAL2, and SRAL1 primers are also depicted.

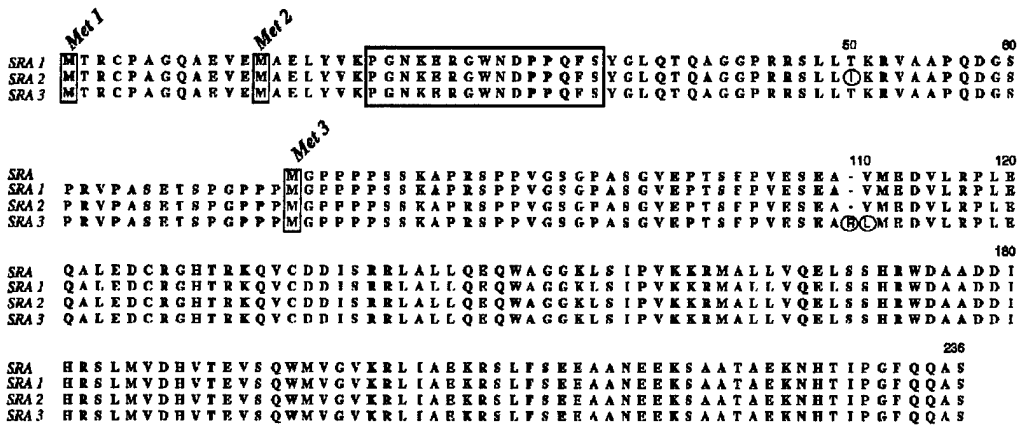

Fig. 3. Alignment of proteins putatively encoded by SRA isoforms. SRA, SRA1, SRA2, and SRA3: proteins putatively encoded by open reading frames contained in AF092038, AF293024, AF293025, and AF293026, respectively. Positions of two putative first methionine (Met 1 and Met 2), together with initially predicted first methione (Met 3, [4]), are indicated. Differences between sequences are circled. Fifteen amino acids highlighted by a box show recognition site for antibody generated against SRA.

Western blot. Protein extracts were separated on SDS–polyacrylamide gels and transferred onto nitrocellulose membranes as previously described [13]. An affinity purified rabbit polyclonal antibody (anti-SRA) raised against the peptide PGNKERGWNDPPQFS (see Fig. 3) was obtained from ResGen (Invitrogen, Carlsbad, CA). Immunodetection of SRA protein was performed using anti-SRA antibody as a primary antibody and a goat-anti-rabbit-HRP conjugated antibody as a secondary antibody as previously described [13]. Antibody neutralization experiments were performed by pre-incubating SRA antibody with its corresponding peptide (2 h, room temperature).

Results

Detection of hypothetical coding SRA isoforms in normal breast tissue

To determine if the hypothetical protein encoded by SRA–mRNA was expressed in vivo, primers were designed corresponding to sequences upstream of the putative first AUG codon (SRAU1) and downstream of the putative stop codon (SRAL1). Total RNA was extracted from two normal breast tissue samples. Reverse transcription and PCR amplification were performed as described in Materials and methods. Using these primers, we obtained the predicted 920-bp PCR product (data not shown). Cloning and sequencing of this fragment revealed that it essentially corresponded to the hypothetical SRA coding sequence (Fig. 2). Interestingly, three different SRA–cDNAs were identified: SRA isoform 1 (GenBank AF293024), SRA isoform 2 (GenBank AF293025), and SRA isoform 3 (GenBank AF293026). SRA isoform 1 contained the full coding hypothetical SRA, whereas SRA isoform 2 contained two point mutations at positions 338 (C → T) and 348 (A → C), and SRA isoform 3 contained a point mutation followed by an insertion of three nucleotides at position 520 (G → CGAC). All these sequences contained a potential open reading frame able to encode two 236 aa and one 237 aa protein for SRA isoforms 1, 2, and 3, respectively (Fig. 3).

In vitro translation of three new SRA isoforms: SRA isoforms 1, 2, and 3

Previously cloned SRA–cDNAs, in which only the third ATG codon (encoding Met 3, Figs. 2 and 3) was present, were unable to support detectable protein synthesis in vitro [4]. To determine if the three new SRA–cDNAs isolated in our laboratory could be translated in vitro, expression vectors containing SRA isoform 1, 2, and 3 sequences downstream of a T7 polymerase promoter were used in a TnT coupled Reticulocyte Lysate system as described in Materials and methods. The three different SRA isoforms encoded stable SRA proteins were produced under these conditions (Fig. 4). Sur-

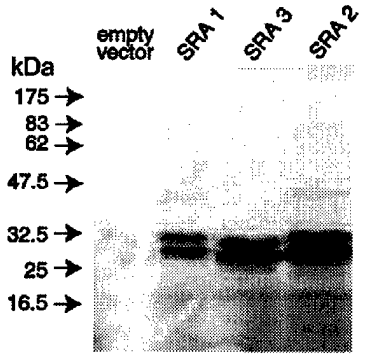

Fig. 4. In vitro translation of SRA isoform 1, 2, and 3 cDNAs. In vitro transcription/translation reactions were performed using SRA isoforms 1, 2, and 3 corresponding expression plasmids and labeled/unlabeled lysates analyzed as described in Materials and methods. Size in kilodalton, corresponding to the prestained marker, broad range (Premixed format, BioLabs), are shown on the left.

prisingly SRA3 encoded protein, even though one amino acid longer is migrating slightly faster than its SRA1 and SRA2 counterparts. It should also be stressed that two bands were observed for each construction, consistent with the possible use of two different initiating methionine codons (Figs. 2 and 3, Met 1 and Met 2). The observed molecular masses (31 and 30 kDa for SRA isoforms 1–2 and 3, respectively) were slightly higher than those predicted (25.7 and 25.8 kDa for SRA isoforms 1–2 and 3, respectively).

Detection of SRA proteins in vivo by transfection of long SRA isoforms

To check whether the newly isolated long SRA isoforms could be translated in vivo, MCF-7 breast cancer cells were stably transfected with expression vectors encoding SRA1, SRA2, or SRA3 protein in frame with a C-terminal V5-His tag. The presence of SRA-tagged proteins in SRA transfected cells only was confirmed by Western blot performed using anti-V5 antibodies (data not shown). As shown Fig. 5A, SRA protein localizes in both the cytoplasm and the nucleus of SRA transfected cells whereas no signal was seen in control cells (MCF7-SRAN cells) transfected with an antisense SRA sequence (Fig. 5B).

Detection of endogenous coding SRA isoforms in breast epithelial cell lines

To determine if the long coding SRA sequences are expressed in breast cancer cells, total RNA was extracted from different breast cancer cell lines and amplified as described. The predicted PCR product corresponding to the hypothetical coding SRAs was observed in all cell lines (Fig. 6A), confirming these transcripts are expressed in tumorigenic and non-tumorigenic human breast epithelial cells. Blast searches of human EST databases (http://www.ncbi.nlm.nih.gov/blast/) revealed that the specific sequence of SRA isoform 3 (i.e., G → CGAC at position 520) had already been cloned by others (GenBank Accession Nos.: AW954396, AW957456, AW 630779, AA305793, AA410852, and AA353911). This confirmed our own data resulting from independent RT-PCRs, cloning, and sequencing and underlined that the SRA isoform 3 was not the result of a technical artifact. Therefore, it was of interest to investigate the expression of this isoform in breast cancer cells. PCR primers were designed to specifically amplify a fragment overlapping the putative insertion region (SRAU2, SRAL2) or to specifically anneal with the inserted sequence (SRAL-**GTCG**) (see Fig. 2 for primer positions). PCR products amplified using the former set of primers (SRAU2, SRAL2) were expected to migrate at an apparent size of 150 and 153 bp for SRA isoform 1–2 and 3 cDNAs, respectively. Using the latter set of primers (SRAU2, SRAL-**GTGC**), a PCR product 117-bp long was expected only in samples expressing SRA isoform 3 mRNA. Results obtained using SRAU2/SRAL2 and SRAU2/SRAL-**GTGC** are shown Figs. 6B and C, respectively. Interestingly, some cell lines expressed only the SRA isoform 3 specific fragment (MDA-MB-231, MCF-7) whereas others expressed both SRA isoforms 1–2 and 3 (T47D, T5).

DNA extracted from these cell lines was also amplified using SRAU2 and SRAL2. As shown in Fig. 6D a

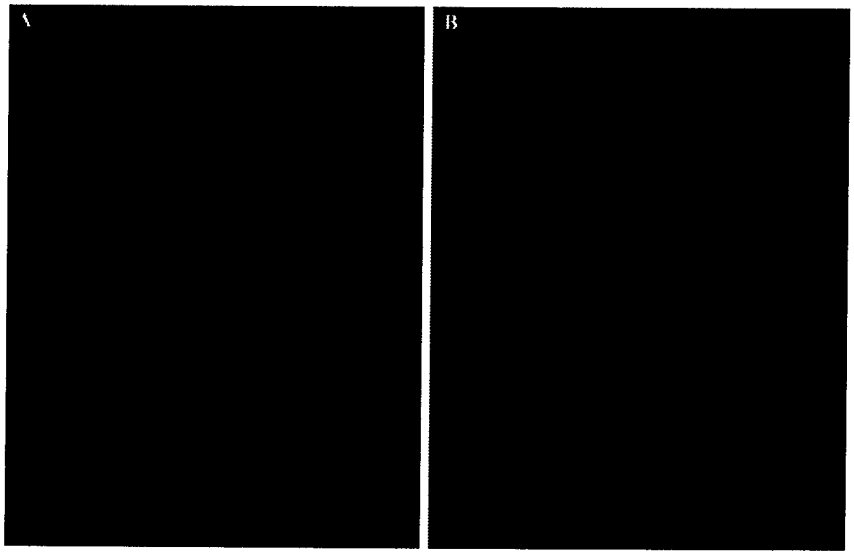

Fig. 5. Detection in vivo of SRA proteins transfected in MCF-7 cell line. MCF7-SRA1 (A) and MCF7-SRAN (B) cells were engineered by stably transfecting MCF-7 breast cancer cells with C-terminal V5-tagged SRA1 sequence or SRA antisense sequence (SRAN). V5 epitope was visualized by immunofluorescence (red signal) and nuclei were counterstained using Hoechst (blue signal) as described in Materials and methods.

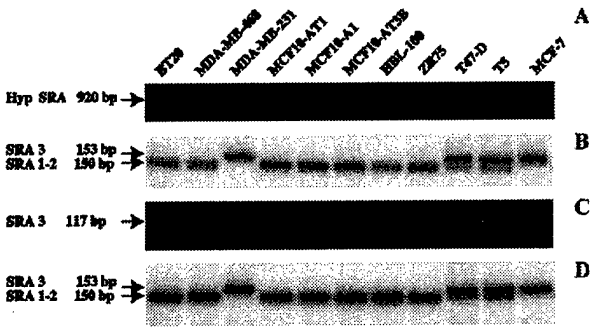

Fig. 6. Detection of SRA isoforms in breast epithelial cell lines. Total RNA was extracted from breast epithelial cell lines (BT-20, MDA-MB-468, MDA-MB-231, MCF10AT1, MCF10A1, MCF10AT3B, HBL-100, ZR-75, T47D, T5, and MCF-7), reverse transcribed, and PCR-amplified using SRAU1/SRAL1 (A), SRAU2/SRAL2 (B), or SRAU2/SRAL-GTCG (C) primers and PCR products were separated on agarose (A, C) or acrylamide (B) gels, as described in Materials and methods. (D) Genomic DNA corresponding to these cell lines was amplified using SRAU2/SRAL2 and PCR products were separated on acrylamide gel as described in Materials and methods.

perfect correlation existed between SRA isoform expression and DNA sequence, suggesting the possible existence of a genetic polymorphism and therefore of at least two alleles of the SRA gene.

Detection of endogenous SRA proteins in breast cancer cells

A rabbit polyclonal anti-SRA antibody was generated and Western blots were performed on breast cancer cells

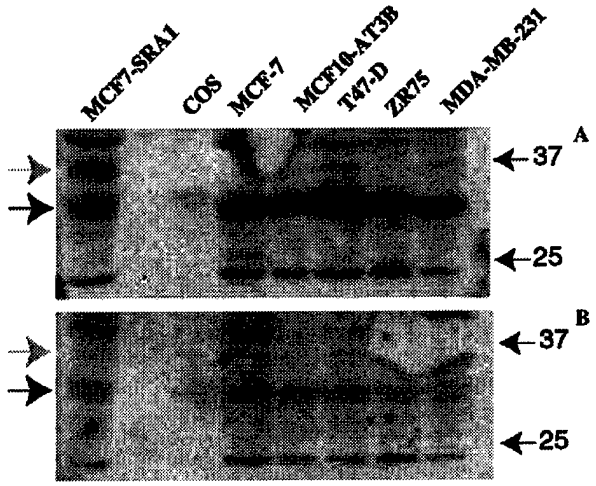

Fig. 7. Western blot detection of endogenous SRA proteins in breast cancer cell lines. Total proteins were extracted from different breast cancer cell lines (MCF-7, MCF10-AT3B, T47-D, ZR75, and MDA-MB-231), separated on SDS–PAGE, and transferred on nitrocellulose membrane. Western blot detection of SRA protein was performed using SRA antibody (A) or SRA antibody neutralized with the corresponding peptide (B), as described in Materials and methods. MCF-SRA1: MCF-7 transfected with SRA1-V5-tagged construction. Cos: Cos cells. Gray arrow: SRA-V5-tagged protein localization, black arrow: endogenous SRA localization.

(Fig. 7) as described in the Materials and methods. A clean signal, absent when the antibody is pre-incubated with the corresponding peptide, is observed at the expected position (~30 kDa) in different breast cancer cell lines. The specificity of the signal observed is further confirmed by the fastest apparent SRA protein migration (SRA3 protein, see Fig. 4) in MCF-7 and MDA-MB-231 cells, shown by RT-PCR to express only SRA3 isoforms.

Discussion

We have identified three new SRA isoforms, longer in their 5′ extremity than those previously described. Database searches revealed that although many partial SRA-like sequences isolated from various normal and tumor tissues have been entered in the human EST sequence database, only a few appear to correspond to these full-length hypothetical protein coding SRA-RNAs. This suggests an overall low expression of these long isoforms or a relative tissue specificity. Interestingly, when investigating SRA-RNA expression by Northern blot Lanz et al. reported the existence of several different sized transcripts expressed in a tissue and cell specific manner. In particular, a 1400 base long SRA-RNA, large enough to contain the minimum 920 bases of our new long SRA isoforms, was strongly expressed in muscle but not in kidney. Such data suggest that the expression of the long hypothetical coding SRA isoforms we described in this study may be tissue specific.

We observed that either one or two SRA isoforms can be expressed in breast epithelial cells. In the situation where two isoforms are expressed at the same time, both alleles are actively expressed. It should be noted that our PCR assays (using SRAU2/SRAL2 and SRAU2/SRAL-GTCG) do not establish whether SRA isoforms 1 and/or 2 are expressed. These assays establish only whether or not SRA isoform 3 is expressed, alone or together with one of the other isoforms (SRA isoform 1 or 2). We found that in breast cancer cells the pattern of SRA isoform expression was directly related to their detection within the genomic DNA. These data suggest the existence of genetic polymorphisms within the SRA gene.

All non-coding SRA isoforms identified by Lanz et al. [4] shared a core sequence starting in exon 2 and stopping in exon 5. This core sequence has been shown to be necessary and sufficient for these RNAs to act as co-activators of steroid receptors. As the longer isoforms we describe here contain this core sequence, it is expected that these latter RNAs could also modify steroid receptor activity as do their shorter previously described counterparts.

Our data are the first to report the detection of an endogeneous SRA protein and to show that naturally expressed human SRA-RNAs can generate a cDNA encoding a detectable SRA protein in vitro and in vivo.

Lanz et al. [4], who reported the identification of three different SRA isoforms, concluded that none of their cloned SRA–cDNA sequences (i.e., predicted to encode a 162 aa protein) could encode a detectable translation product. Interestingly, when engineered to form a fusion protein, e.g., with GST, Gal4 or HSV-thymidine kinase initiation sequences attached to the N-terminal region, the appropriate fusion SRA-like product was detected. Taken together, these data suggest that the 5′ extremity of their cloned SRA was missing a functional initiating methionine codon or that the extra 74 N-terminal amino acids predicted in our hypothetical coding SRA–mRNAs are required for the stable expression of the resulting protein. This is reflected by the production of two different sized proteins in vitro and in vivo from each of our isoforms, presumably because of a choice in initiation start sites. Interestingly, during the preparation of this manuscript, Karashiwa et al. [16] reported the translation in vitro of a rat SRA related molecule (SRAP). When comparing SRAP protein sequence with our SRA1 protein (entered into GenBank in 2000), these authors observed a 78% conservation in amino acid sequence identity in the 146 amino-acid C-terminal region of SRA1 protein. The putative SRAP in the rat is much smaller (16 kDa) than the human SRA proteins we describe here. Karashiwa et al. were able to express in vivo a rat SRAP protein when fused with the C-terminal extremity of GST or GFP. However, these authors have still to prove the stable expression in vivo of an SRAP protein from its own naturally occurring initiation AUG codon, as well an endogenous rat SRAP protein. Of considerable interest is the function described for this SRAP protein. Kawashima et al. reported that SRAP directly interacted with the androgen receptor (AR) and the glucocorticoid receptor (GR) to increase the transcriptional activity of these receptors. As the human SRA proteins share a strong sequence homology with the rat SRAP in their C-terminal domain, we hypothesize that human SRA protein could also interact with and modify the activity of steroid receptors, as do their RNAs. The nuclear localization of SRA protein suggests the possibility of such an interaction. Further experiments are needed to clarify the exact function of these new long SRA isoforms and their encoded proteins. However, if our latter hypothesis is confirmed, SRA might become the first molecule to be active in the same signalling pathway both at the RNA and at the protein levels.

Acknowledgment

E.L. is the recipient of a USAMRMC Career Development Award Grant No. DAMD17-01-1-0308.

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FEBS 28354

FEBS Letters 566 (2004) 43–47

The steroid receptor RNA activator is the first functional RNA encoding a protein

S. Chooniedass-Kothari[1], E. Emberley[1], M.K. Hamedani, S. Troup, X. Wang, A. Czosnek, F. Hube, M. Mutawe, P.H. Watson, E. Leygue*

Department of Biochemistry and Medical Genetics, Faculty of Medicine, University of Manitoba, P309 Pathology Building, 770 Bannatyne Avenue, Winnipeg, MB, Canada R3E 0W3

Received 11 March 2004; accepted 21 March 2004

Available online 20 April 2004

Edited by Robert Barouki

Abstract The steroid receptor RNA activator (SRA) has previously been characterized as belonging to the growing family of functional non-coding RNAs. However, we recently reported the Western blot detection of a putative endogenous SRA protein (SRAP) in breast cancer cells. Herein, we successfully suppressed the expression of this protein through specific RNA interference assay, unequivocally confirming its existence. Moreover, using database searches and Western blot analysis, we also showed that SRAP is highly conserved among chordata. Overall, our results suggest that SRA is the first example of a new class of functional RNAs also able to encode a protein.

Keywords: Steroid receptor RNA activator; Non-coding RNA; RNA interference; Steroid receptor RNA activator homologue; Steroid receptor RNA activator protein

1. Introduction

Even though RNAs have long been thought to be either messenger RNAs (mRNAs), transfer RNAs or ribosomal RNAs, it has become apparent in the last 20 years that many RNAs do not belong to any of these three subgroups [1]. The family of non-coding RNAs (ncRNAs), which groups all RNAs unable to encode a protein, is increasing exponentially [2]. Data for the new members of this growing family are actively gathered and corresponding information sorted on several websites such as http://biobases.ibch.poznan.pl/ncRNA/, http://rfam.wustl.edu/index.html or http://indiana.edu/~tmrna/. These RNAs which have no protein coding capacity have been shown to regulate several cellular processes as diverse as the subcellular distribution of RNAs and proteins, the modulation of protein function, or the transcriptional and translational regulation of gene expression [1,2]. The steroid receptor RNA activator (SRA) has recently been characterized as one such ncRNA that modulates steroid receptor transcriptional activity [3].

The originally described SRA sequences differed in their 5′ and 3′ ends, but were conserved in their central core region [3]. The core region was shown to be necessary and sufficient to increase the ligand-dependent transcriptional activation of target genes by steroid receptors. None of these original SRA sequences were successfully translated in vitro or in vivo [3], and SRA is still currently classified as belonging to the expanding family of functional ncRNAs [4]. Since 1999, data have accumulated regarding the possible mechanisms of action of SRA RNA. SRA RNA interacts with other proteins such as the co-repressor Sharp and the AF-1 specific activator p72/p68 protein to modulate steroid receptor activity [5,6]. Moreover, SRA RNA potentiates the estrogen-induced activation of both estrogen receptors α and β [7]. By introducing mutations in the SRA RNA sequence, Lanz et al. recently identified motifs participating in the RNA secondary structure that are involved in its ability to co-activate progesterone receptor [8,9].

We recently identified three new SRA RNA isoforms which corresponded to SRA except for an additional 37 nucleotides in the 5′ region [10]. This 5′ region contains two putative ATG codons, close together in the same open reading frame, that could encode putative 236/224 amino-acid SRA proteins (SRAPs). These isoforms, which contain the functional core region, encoded a stable protein both in vitro and in vivo. Using reverse polymerase chain reaction of RNA extracts, we were able to confirm the presence of these endogenous coding isoforms in breast cancer cell lines [10]. Furthermore, using an antibody raised against a peptide corresponding to amino acids 20–34 of the putative human SRAP, we were able to specifically detect a doublet at 30 kDa by Western blot analysis of total protein lysate from these same cell lines [10].

To date, all other reports describe and discuss human SRA as a ncRNA molecule. To our knowledge, no functional RNA has been described to have a protein coding capacity. It, therefore, became important to confirm unequivocally the existence of such an SRAP. In the present study, we demonstrate that the human SRA gene not only encodes for a protein but that the sequence of this protein is conserved among vertebrates.

* Corresponding author. Fax: +204-789-3900.
E-mail address: eleygue@cc.umanitoba.ca (E. Leygue).

[1] These authors have equally contributed.

Abbreviations: SRA, steroid receptor RNA activator; RNAi, RNA interference; ncRNA, non-coding RNA; SRAP, steroid receptor RNA activator protein

0014-5793/$22.00
doi:10.1016/j.febslet.2004.03.104

2. Materials and methods

2.1. RNA interference (RNAi) vector construction and SRAP knockdown assay

pSuper.retro-SRA construct was generated by hybridization of oligonucleotides SRARNAif (5′-ccccaagtttccctcacgagtcttcaagagagactcg actgggaaacttgtttt-3′) and SRARNAir (5′-aaacaagtttcccagtcgagtctctctt gaagactcgactgggaaacttgggg-3′), which were then cloned between the *Bgl*II and *Hind*III sites of the pSuper.retro vector (Oligoengine, Seattle, WA). The SRARNAi oligonucleotide sequence was selected following analysis with the Oligoengine RNAi design tool (http://www.oligoengine.com/). HeLa cells were transiently transfected with either the pSuper.retro-SRA construct or pSuper.retro vector (empty vector) using the transfection agent Effectene (Qiagen, Missisauga, ON) according to the manufacturer's protocol. At indicated times, cells were washed in 1× PBS and lysed with 2× SDS buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 0.1% bromophenol blue and protease inhibitor cocktail (Roche, Laval, QB). Protein sample concentration was determined by the Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Protein samples were analyzed by Western blot.

2.2. Western blot analysis

Proteins were extracted from skeletal tissue [11] or cell lines [10] as described previously. We chose total skeletal muscle as Lanz et al. [3] had shown that SRA RNA is highly expressed in human skeletal muscle. Protein concentration for each sample was determined using Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) and equal amounts of total protein were analyzed by SDS–PAGE and immunoblotting. Supernatant samples containing equal amount of total protein were mixed 1:1 with sample buffer [1.25 mM Tris–HCl (pH 6.8), 20% glycerol, 4% SDS, 0.02% (w/v) bromophenol blue and 20 mM DTT], boiled for 5 min, electrophoresed using a 5% stacking gel and a 10% resolving polyacrylamide gel, and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (pH 7.6) and incubated with rabbit polyclonal antibodies raised against amino acids 20–34 of human SRA sequence [10] at a dilution of 1:1000 in 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween. Secondary horseradish peroxidase-linked goat antirabbit antibodies (1 μg/μl, Bio-Rad) were then used and signals were analyzed by SuperSignal West Pico Chemiluminescent Substrate (Pierce). Chemiluminescence signal was captured by video image analysis using the Quantity One system (Bio-Rad). To determine equal loading, the SDS–PAGE gels were either stained in Coomassie blue solution (50% methanol, 10% acetic acid and 0.1% w/v Coomassie powder) following transfer or blots were stripped and reprobed with anti-β-actin antibody (Sigma, Oakville, ON). In order to detect the specificity of the detection in the protein extracts from different species, we incubated duplicate blots with anti-SRA antibody premixed with the peptide used to raise it (1/10 v/v).

2.3. Sequence analysis and database searches

Search of the NCBI protein database (www.ncbi.nlm.nih.gov) led to the identification of the SRA sequences for three species: *Homo sapiens* (GenBank Accession Nos. AF293024, AF293025 and AF293026), *Mus musculus* (GenBank Accession No. NP_079567) and *Rattus norvegicus* (GenBank Accession Nos. NP_000035 and AAG02116). Upon examining the mouse and rat expressed sequence tag (EST) database, a longer 5′ *M. musculus* sequence (GenBank Accession No. CB274276) and a *R. norvegicus* sequence (GenBank Accession No. CB771552) were identified. These sequences were used to deduce the theoretical sequences for the rat and mouse SRAPs. Search of the Unigene database and ProtESTs (http://www.ncbi.nlm.nih.gov/UniGene/protest.cgi?SORT=4&ORG=Hs&XID=114234) led to the identification of SRA EST sequences for the following species: *Xenopus laevis* (GenBank Accession Nos. BG364002.1, BG551872.1 and AW642449.1), *Silurana tropicalis* (GenBank Accession No. AL969036.1), *Sus scrofa* (GenBank Accession Nos. CF366666 and CF368085), and *Oryzias latipes* (GenBank Accession No. AU170197.1). We blasted the human SRA3 amino-acid sequence against the translated EST nucleotide sequences using tblastn, limiting the search to vertebrates and excluding mouse and human sequences. This search identified ESTs for the following additional species: *Macaca mulata* (GenBank Accession No. CD766957), *Equus caballus* (GenBank Accession Nos. B1961443 and B1961063), *Bos taurus* (GenBank Accession Nos. CB422540, AW654516, CB450664 and CB457765P), *Danio rerio* (GenBank Accession Nos. BQ258955, CB352395), *Occhonrhyncus Mykiss* (BX860673) and *Gallus gallus* (GenBank Accession Nos. CR338992, CR338823, CR338795). Species-specific sequences were assembled using an EST assembler tool (http://bio.ifom-firc.it/ASSEMBLY/assemble.html) giving contigs that were translated using the DNA to amino-acid translational tool (http://ca.expasy.org/cgi-bin/dna_aa.html). All the putative SRAP sequences were aligned using the Multalin alignment tool (http://prodes.toulouse.inra.fr/multalin/) and two conserved regions were identified. These two conserved sequences were used to identify the SRA analogue in Fugu fish by doing a Blast search against the Fugu fish genome. Blast search using the human SRA1 sequence against the ascidian *Ciona intestinalis* genome in the TIGR database (http://tigrblast.tigr.org/tgi/) resulted in the identification of an SRA analogue (GenBank Accession No. BW276199) in *C. intestinalis*. Blast searched at the NCBI site http://www-genome.wi.mit.edu/cgi-bin/annotation/ciona/blast_page.cgi using the *C. intestinalis* SRA sequence resulted in a *Ciona savignyi* SRA analogue. Nuclear localization signals were search on each individual sequence using Psort II (http://psort.nibb.ac.jp/form2.html).

2.4. PCR cloning and in vitro translation of rat SRA cDNA

PCR primers (5′-agtgagctaccaccccggaa-3′ and 5′-tatagaagtcatgtgaggt-3′) designed by analyzing the rat theoretical SRA sequence were used to amplify cDNA from rat skeletal muscle. The resulting product was sequenced (GenBank Accession No. AY542868) and cloned in pcDNA3.1 expression vector. RNA isolation from rat skeletal tissue and reverse transcription-polymerase chain reaction were conducted as described previously [9]. pcDNA3.1 (Invitrogen, Carlsbad, CA) expression plasmid containing either human SRA cDNA or rat SRA cDNA was used for in vitro translation/transcription reaction. [^{35}S]methionine labelled SRAPs were generated using wheat germ lysate coupled transcription/translation reactions by the TnT System (Promega, Madison, WI) according to the manufacturer's instructions. The V5-tagged human SRA cDNA construct, previously [10] cloned in pcDNA3.1/V5-His© (Invitrogen, Carlsbad, CA), was used as a positive control. Lysates were then subjected to SDS–PAGE separation, after which gels were dried and [^{35}S]methionine labelled protein bands visualized by exposing overnight to a Molecular Imager™-FX Imaging screen (Bio-Rad) and subsequently scanned using a Molecular Imager™-FX (Bio-Rad).

3. Results and discussion

We were previously able to detect a putative endogenous SRAP in breast cancer cell lines with an antibody targeted against amino acids 20–34 of the hypothetical human SRAP sequence [10]. As we are still today the only ones to have reported the existence of such a putative protein, it became essential to definitively confirm the identity of the protein recognized by our antibody. In order to do so, we used the recently developed RNAi technology in an attempt to knockdown its expression. Indeed, we reasoned that the specific degradation of SRA RNA should result in a decrease in the expression of the protein recognized by our antibody. Upon 24 and 48 h, transfection of HeLa cells with a RNAi specifically targeted against SRA RNA resulted in a significant decrease in the doublet detected by Western blot and believed to correspond to the SRAP (Fig. 1A). In contrast and as expected, RNAi treatments had no significant effect on β-actin levels (Fig. 1B) as well as a non-specific protein detected by our anti-SRA antibody (Fig. 1A, 45 kDa). This result links the expression of the SRA gene to the detection of the suspected SRAP by Western blot. This unequivocally confirms for the first time the existence of an endogenous SRAP in human cells. It should be stressed that no noticeable phenotypic changes were observed in cells treated with SRA-specific RNAi over the 48-h post-transfection. This absence of apparent effect

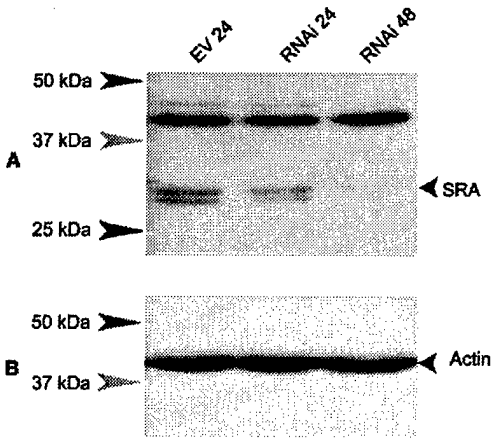

Fig. 1. Decrease of SRAP expression by RNAi specifically targeted against SRA RNA. HeLa cells were transiently transfected with either SRA RNAi (RNAi) or control (EV) vectors, and SRA or actin protein expressions assessed by Western blot 24 and 48 h after transfection as described in Section 2. Positions of the molecular size markers are indicated on the left.

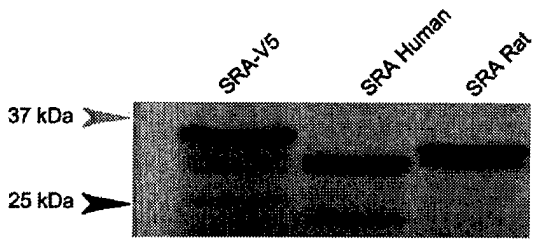

Fig. 2. In vitro translation of human and rat SRA cDNAs. In vitro transcription/translation reactions were performed using V5-tagged human SRA cDNA as control (SRA-V5), human SRA cDNA (SRA Human) and rat SRA cDNA (SRA Rat) as described in Section 2. Positions of the molecular size markers are indicated on the left.

likely results from the short length of time and the transient nature of these experiments have been conducted. Further experiments are needed to investigate longer term effects of the knockdown of SRA gene on phenotypic changes such as growth rate.

We have then investigated the possible existence of this SRAP in other species. As of today, Blink, the NCBI sofware which groups putative species homologues (http://www.ncbi.nlm.nih.gov/sutils/blink.cgi?pid=9930614), only gathers human, rat and mouse SRA sequences. Rat SRA sequences (GenBank Accession Nos. NP_000035 and AAG02116) correspond to the recently published 146 amino-acid rat SRAP sequence [12]. This putative rat SRAP sequence was successfully translated in vitro and also expressed in vivo when fused with the C-terminal extremity of green fluorescence protein. SRAP is much smaller (16 kDa) than the putative human SRAP we identified and its endogenous existence has not yet been demonstrated. Close analysis of the SRAP mRNA sequence revealed that it is analogous to the human SRA sequences starting at exon 2. We thus suspected the SRAP mRNA sequence described so far to be incomplete. Indeed, analysis of the rat chromosome 18 sequence revealed the presence of a putative additional exon 1 present in a single rat EST sequence (GenBank Accession No. CB771552). To confirm the existence of this longer SRA sequence, we PCR amplified rat muscle cDNA with specific primers as described in Section 2. We cloned a new rat cDNA sequence (GenBank Accession No. AY542868), which unlike the shorter SRAP sequence contains two possible methionines possibly initiating the translation of a 222/230 amino-acid proteins. As shown in Fig. 2, this sequence is translatable in vitro, generating a visible doublet at 31/32 kDa. As expected and as a result of an additional V5-tag (4.8 kDa), in vitro translated SRA-V5 protein (35 kDa) has a higher apparent molecular size than the non-tagged human SRA doublet (30/31 kDa) and rat SRA doublet (31/32 kDa). As previously described [10], the observed molecular masses (35, 30/31 and 31/32 kDa) are slightly higher than those predicted (30.5, 25.7 and 25.3 kDa for the V5-tagged human, non-tagged human SRAP and rat SRAP, respectively). We hypothesize that the doublet seen for human and rat SRAPs results from the alternative use of one initiating methionine instead of another (1 versus 12). Weaker lower molecular size bands (around 25 kDa) are likely resulting from translation at internal downstream methionines present in both the human and rat SRA sequences (see Fig. 3).

Through database analysis, we identified a *M. musculus* sequence (GenBank Accession No. CB274276), slightly longer than the one present in the protein database. When translated, this sequence contains an additional stretch of 12 N-terminal amino acids, 10 out of which are identical to their corresponding human counterparts (Fig. 3).

In an attempt to identify SRA analogues in other species, we searched several databases as described in Section 2. Obtained cDNA sequences were translated and aligned (Fig. 3). The alignment of putative SRA sequences from different species shows proteins of similar lengths, highly conserved in discrete domains and in two main regions (amino acids 15–39 and 180–208 of the human sequence). We have previously demonstrated that the stably transfected SRAP localizes to the nucleus in MCF-7 cells [10]. Analysis of the SRA sequences (Psort II), for nuclear localization signal domains, revealed a conserved pat-7 (P-x-[RK]-[RK]-[RK]) nuclear localization motif in *Gallus* (chicken), *Oryzias* and *Occonrhyncus* (two fish) and all the mammalian putative SRAP sequences (amino acids 155–160). Interestingly, the two *Ciona* species which do not contain pat-7 motif at this position, contain however another nuclear localization pat-4 motif (P-[RK]-[RK]-[RK]) at amino acids 39–42 with respect to the human SRA sequence.

Overall, putative SRAPs were found in all vertebrates in which SRA-related EST sequences could be detected. In addition, putative SRAP analogues were identified in two *Ciona* species, *Ciona savignyi* and *C. intestinalis*. *Ciona* belong to the urochordata subphylum, which together with Cephalochordata and Craniata (contains Vertebrata) subphyla, defines the *Chordata phylum*. We were unable to find any sequences closely related to SRA in any other phyla such as Arthropoda (*Drosophila melanogaster*), Nematoda (*Caenorhabditis elegans*) or Protobacteria (*E. coli*). Conservation of the SRAP sequence from an invertebrate Chordata (*Ciona*) to a higher vertebrate (human) suggests an important role possibly played by this molecule. Furthermore, the conservation of a nuclear localization signal in most of the SRAPs suggests a nuclear localization for this putative conserved function. In addition, the apparent absence of SRA homologues in all non-chordata phyla suggests that this protein might have been involved in the emergence of early Chordata.

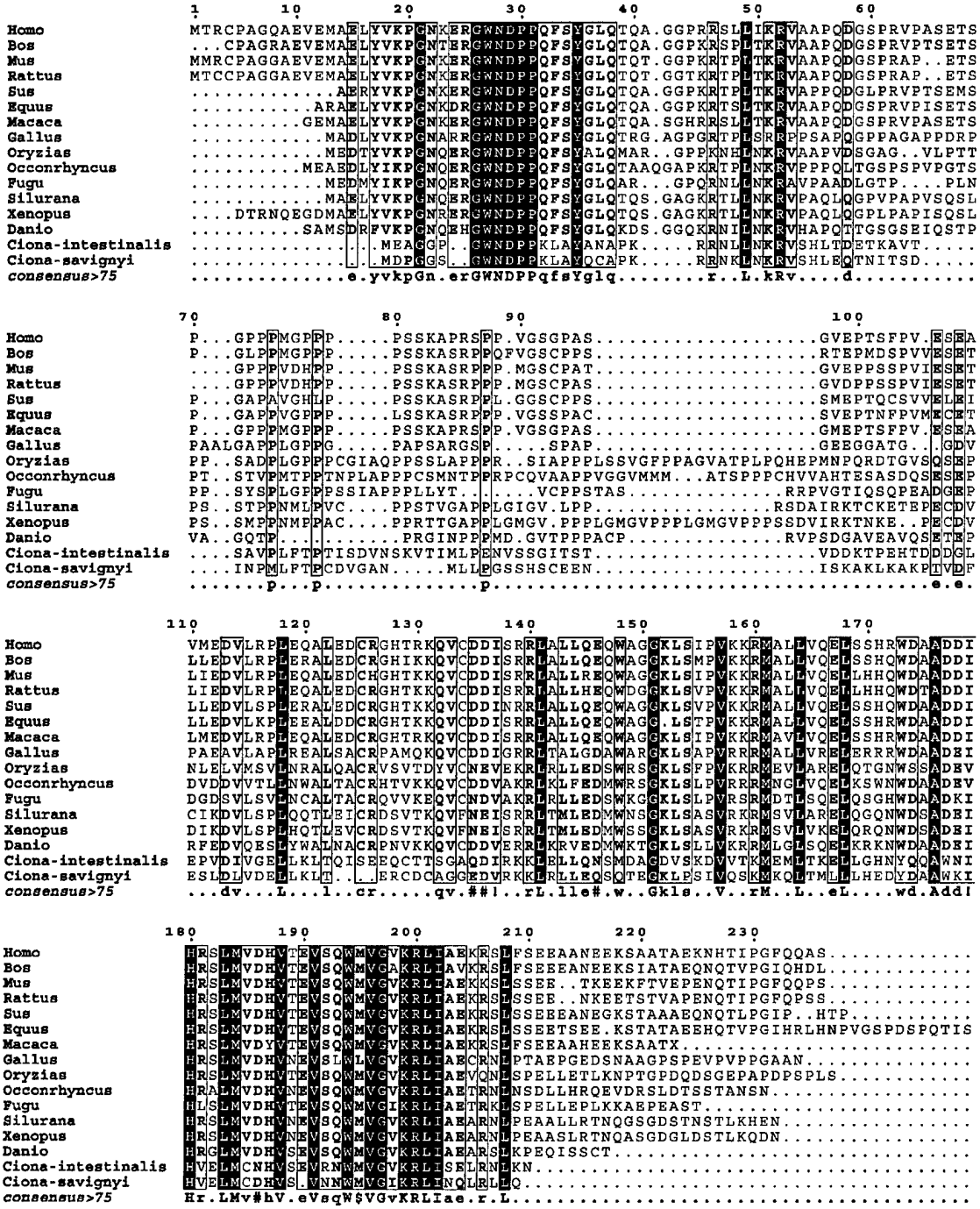

Fig. 3. Alignment of putative SRAP sequences from several species. SRAP homologues were found as described in Section 2 for the following species: *H. sapiens*, *B. taurus*, *M. musculus*, *R. norvegicus*, *S. scrofa*, *E. caballus*, *M. mulata*, *G. gallus*, *O. latipes*, *O. mykiss*, *Fugu rubripes*, *S. tropicalis*, *X. laevis*, *D. rerio*, *C. intestinalis* and *C. savignyi*. The numbers indicated on top of the alignment correspond to amino-acid sequence of the human SRA isoform 1. Regions conserved in all species are in dark bold. Boxed regions correspond to partially conserved sequences.

As most of the putative sequences obtained were well conserved in the region between amino acids 15 and 39, we hypothesized that our antibody, raised against the peptide 20–34 of the human SRAP sequence, could recognize SRAPs from other species. Western blot analysis of SRAP expression was therefore performed on protein extracted from skeletal muscles (tissue known to contain high levels of SRA RNA [3]) of several non-human vertebrates.

As shown in Fig. 4A, we were able to specifically detect a band of similar size (around 32 kDa) in *Bos* (cow), *Sylvilagus* (rabbit), *Sus* (pig), *Gallus* (chicken), *Meleagris* (turkey) and *Ovis* (sheep). Equal amounts of total protein extracts were

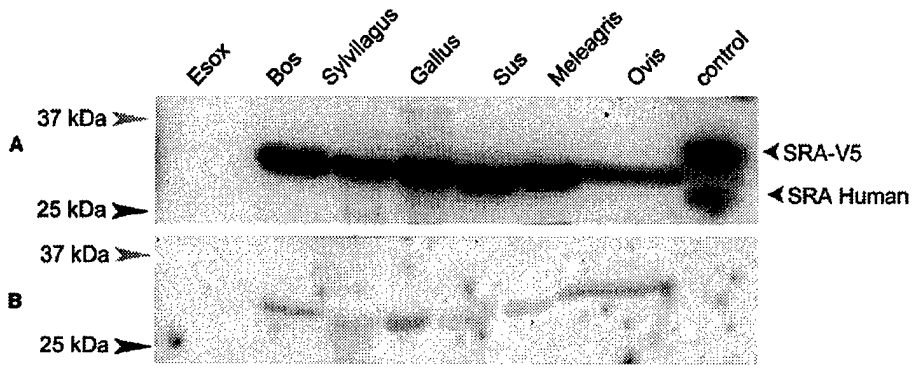

Fig. 4. SRAP expression in skeletal muscle of various species. As described in Section 2, Western blots of total protein extracts from the muscle of different species were performed in the absence (A) or presence (B) of neutralizing peptide. Positive control consists of protein extracts from MCF-7 cell line stably expressing V5-tagged human SRA. Positions of the molecular size markers are indicated on the left.

loaded as confirmed by Coomassie blue staining of the gels after transfer (data not shown). Protein extracts from MCF-7 cells stably expressing V5-tagged SRA were used as positive control (right lane). An upper band corresponding to the transfected V5-SRA is seen at 35 kDa. In addition, a doublet, corresponding to the endogenously expressed SRA is seen at 30/31 kDa. No signals were observed when the antibody was pre-neutralized by the corresponding peptide (Fig. 4B). Interestingly, only one band is detected in muscle extracts, suggesting that only one AUG codon is used to initiate SRAP translation in these tissues. This may result either from the existence of only one such codon in the *Sus*, *Bos*, *Gallus* putative mRNA sequences (see Fig. 3), or from the preferential tissue-related use of one codon over the other.

No SRAP was detected in the Esox (pickerel) protein extract. A careful examination of the four hypothetical fish sequences we gathered (*Fugu*, *Oryzias*, *Occhonrhyncus* and *Danio*) shows that they are similar to the human sequence in the region recognized by the antibody used (20–34, referenced to human SRA) except for the presence of a glutamine instead of a lysine at amino acid 23 (Fig. 3). The Esox SRA sequence, which remains unknown to date, should, if similar to the other fish sequences in this particular region, also contain this particular amino-acid substitution. Interestingly, our antibody was able to recognize SRAPs from the *Bos* (cow) and *Gallus* (chicken) tissue extracts despite the presence of a threonine (*Bos*) and an alanine (*Gallus*) instead of a lysine at this same residue 23. Lysine, alanine and threonine as opposed to glutamine are all hydrophobic amino acids. We therefore suspect that a switch in the hydrophobicity of the amino acid at residue 23 may be responsible for the impaired recognition of Esox SRAP by our antibody.

In this study, we have demonstrated that the human SRA gene encodes for a protein conserved among vertebrates. The high conservation of SRAP sequence underlines the possible important role played by this protein. Previous studies have shown that SRA RNA is fully functional independently of its protein coding capacity. SRA therefore appears to be the first example of a new class of functional RNAs also able to encode a protein.

The existence of an SRAP raises several important questions waiting to be addressed. What is the role of SRAP? What are the implications of SRAP on SRA RNA function? How are the expressions of SRA RNA and SRAP regulated? What other molecules function at dual protein/RNA levels? Since all of the functional studies on human SRA described to date ignore the existence of a protein, addressing the above questions is critical to fully understand SRA function. More importantly, development in our understanding of SRA RNA and protein function is in turn critical for a change in the current perspective of functional "non-coding" RNA molecules.

Acknowledgements: This work is supported by a USAMRMC Career Development Award Grant No. DAMD17-01-1-0308 and a CBCRA Grant No. 12326.

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Appendix 13 Abstract #2872. AACR meeting 2004

Abstract Number: 2872

The steroid receptor RNA activator (SRA) encodes a protein conserved in vertebrates

Shilpa K. Chooniedass, Sandra Troup, Mohammad K. Hamedani, Magdalena Drewniak, Anna Czosnek, Xuemei Wang, Etienne Leygue. University of Manitoba, Winnipeg, MB, Canada.

Steroid receptor RNA activator (SRA) is a steroid receptor co-activator that has recently been shown to enhance estrogen induced transcriptional activity of estrogen receptors alpha and beta. SRA differs from the other steroid receptor co-activators, in that it was shown to function as a non-coding RNA molecule. The initial paper on SRA reported a lack of success in generating any in vitro or in vivo stable SRA protein. We have identified three SRA RNA isoforms sequences which, unlike the previously described non-coding RNA, have a longer 5' end and encode a stable SRA protein. Through database analyses, we have also identified mRNA sequences of SRA for several classes of vertebrates. Analysis of the protein sequences showed that the SRA protein family members are similar in size and have two extremely well conserved regions. Using an SRA antibody targeted against the N-terminal conserved region of the protein and muscle extracts from different vertebrate species, we have detected in a protein band of similar size. In MCF-7 cells stably transfected with SRA cDNA, we unexpectedly found that ER alpha activity is reduced in the presence of estradiol compared to non-transfected cells. Therefore our results suggest a possible role for SRA protein that is distinct from the SRA RNA.

Presenter: Shilpa K. Chooniedass

Affiliation: University of Manitoba, Winnipeg, MB, Canada; E-mail: kothari_shilpa@hotmail.com

 Citation information: Proceedings of the AACR, Volume 45, March 2004.

Appendix 14 Abstract, submitted San Antonio Breast Cancer Meeting, 2004

Title: Expression of the newly described steroid receptor RNA activator protein (SRAP) in breast tumor tissue. Shilpa Chooniedass-Kothari, Sandy Troup, Mohammad K Hamedani, Xuemei Wang and Etienne Leygue. [1] Biochemistry and Medical Genetics,

University of Manitoba, Winnipeg, Manitoba, Canada, R3E 0W3 .

Body: The steroid receptor RNA activator (SRA) has originally been described as the first functional non-coding RNA able to specifically co-activate steroid receptors. We have however recently demonstrated the existence of a corresponding protein, SRAP, in breast cancer cell lines. Today, there is no functional data available on any possible role of human SRAP. In order to address the possible effects of SRAP on the estrogen receptor signaling pathway, we generated an estrogen receptor positive breast cancer cell line MCF-7-SRAP, stably over-expressing this protein. Transient transfection of these cells by a luciferase reporter gene under the control of an estrogen responsive element ERE revealed a decreased sensitivity to estradiol (figure 1). Interestingly, Western blot analysis of SRAP expression in 74 primary breast tumors of patients subsequently treated with Tamoxifen showed that SRAP positive patients (n=24) had a significant (Kaplan Meir survival curve, p = 0.047) lower likelihood to die from recurrent disease than SRAP negative patients (n=50) (figure 2). Overall, our data suggest that SRAP protein could not only modify the activity of the estrogen receptor signaling pathway in breast cancer cells but could also be a marker in primary tumor of a subsequent better response to tamoxifen treatment.

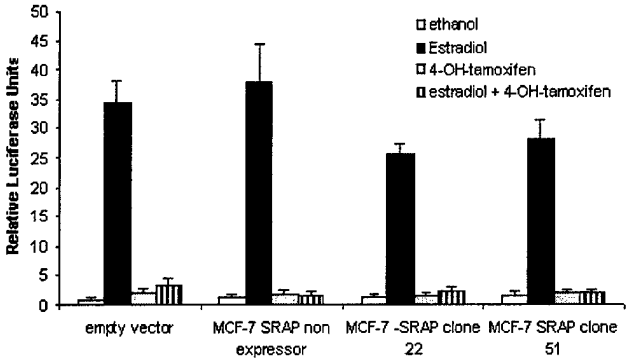

Figure 1. Effect of SRAP expression on estrogen receptor activity upon estradiol (10^{-8}M) and tamoxifen (10^{-6}M) treatments.

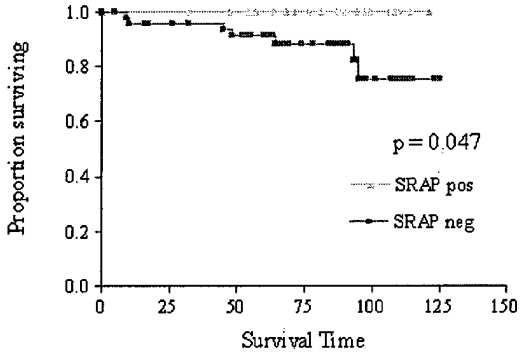

Figure 2. Overall survival of patients subgroups with or without SRAP expression (74 ER positive patients treated with tamoxifen).